ACTIVITIES OF ANTI-LIPOPOLYSACCHARIDE IMMUNOGLOBULINS.

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ABSTRACT.

Several aspects of the activities of anti-lipopolysaccharide (anti-LPS) immunoglobulins were assessed in relation to their potential for therapeutic use in Gram-negative septicaemia.

i) The binding activities of immunoglobulins present naturally in human blood donor sera to a wide range of LPS antigens were assessed in an ELISA system. This assay incorporated LPS-polymyxin complexes as antigens. There was wide variability in antibody reactivity to different LPS both within and between individuals. ELISA was also performed on IgG purified from donor sera. Immunoblotting with sera or IgGs failed to produce results comparable to those in ELISA.

ii) Assessment was made of a putative relationship between anti-core glycolipid (anti-CGL) antibodies and levels of endotoxin during episodes of septic shock using ELISA and Limulus amoebocyte lysate (LAL) assays. Results were complex but indicated an inverse relationship between anti-CGL antibodies and endotoxin activities in serum.

iii) Long-term immunisation of rabbits was carried out to determine immunoglobulin responses to smooth LPS (S-LPS), rough LPS (R-LPS) and lipid A. Six rabbits were each administered with a different range of smooth or rough bacteria and antibody response was assessed by ELISA. A complex series of responses was obtained, but antigenic relationships between some LPS molecules was indicated.

iv) Antigenic expression of R-LPS was assessed by ELISA with 4 different antigen preparations of S. typhimurium R878 (LPS-polymyxin complexes, uncomplexed LPS, outer membrane fragments, and heat-killed bacteria). Absorption and inhibition studies indicated that antigenic expression of LPS in all preparations was similar.

v) Alteration of lipopolysaccharide was shown to occur in two isogenic variants of E. coli (O18:K1 and O18:K^-). Variations were observed in different media and at different points during growth. Differences were also observed in the binding activities of core- and O-antigen-reactive monoclonal antibodies under these conditions. The anti-O-antigen antibody bound to bacteria grown under all conditions, whereas the core-reactive monoclonal antibody bound to both variants when grown in untreated sheep serum and at low levels to only the non-capsulate variant when grown in heat-inactivated serum.

vi) Endotoxin neutralising activity of 5 purified IgG was determined in a LAL inhibition assay. Seven lipopolysaccharides were used as activators and all IgG were shown to possess some inhibitory activity against LPS. Extent of inhibition was not reflected by ELISA profiles, and possible reasons are discussed.

vii) Several human immunoglobulin preparations were assessed for their ability to prevent death in a range of animal models of bacteraemia and endotoxaemia. A range of non-compromised and immuno-compromised models were used to determine lethality of purified LPS and viable bacteria in the presence and absence of immunoglobulins. Some indications were obtained of protection against lethal bacterial challenge, and thus of the potential therapeutic value of selected human immunoglobulin products in patients with septicaemia.

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DECLARATION

All of the investigations and procedures presented in this thesis were performed by the author unless indicated otherwise in the aknowledgements.

INTRODUCTION

1:1. Aetiology and Epidemiology of Gram-negative Septicaemia.

1:1:1. Causative Organisms of Gram-negative Septicaemia.

The advent of antimicrobial agents during the 1930s and 1940s and their subsequent application, together with the improvement of care and advancement of therapeutic drugs has enabled severely ill patients, who would previously have died, to survive. This has consequently introduced the possibility of infection by bacteria not previously recognised as primary pathogens. That is, opportunistic infection may occur as a result of the compromised state of these individuals. It is in this recently developed niche that aerobic (and to a lesser extent anaerobic) Gram-negative rod-shaped bacteria have become predominant as causes of life-threatening infections, especially in hospital patients, despite the application of antimicrobial agents.

Prior to the advent of antimicrobial agents, Gram-negative bacteria were known as primary pathogens causing, for example, brucellosis, pneumonia, salmonellosis, and plague (Weinstein 1985; Young 1985b; Young 1985c). These diseases were transmitted via animal or insect vectors or in food and water, and possess an obviously different mode of pathogenicity to the recently emerged group of Gram-negative opportunist pathogens.

The incidence of nosocomial (hospital-acquired) infection caused by both Gram-negative and Gram-positive organisms has been increasing since the 1940s (Alford & Hall 1987; Cone & Woodward 1985; Haley *et al* 1985; Mertens *et al* 1987; McGowan 1985; de la Torre *et al* 1985)

in step with the increasing use of antimicrobial agents (Mayer & Zinner 1985; Neu 1985; Young 1985c). Many of these infections are caused by invasion of the blood-stream by bacteria, and the rate of septicaemia found in hospitals has been shown to be up to 20 per 1000 patients admitted (summarised in Table 1:1).

TABLE 1:1. Incidence of Nosocomial Bacteraemia.

Cases of Bacteraemia per 1000 patients	Country	Reference
7.1	(U.K.)	Ispahani *et al* 1987
2.8	(Denmark)	Eliasen *et al* 1987
15.0	(Sweden)	Julander 1987
13.2	(Spain)	Bisbe *et al* 1988
2.3	(Spain)	de la Torre *et al* 1985
19.1	(Spain)	Gatell *et al* 1988
13.5	(Israel)	Seigman-Igra *et al* 1988
7.6 (*E. coli* only)	(U.S.)	Bodey *et al* 1985
4.5 (*P. aeruginosa* only)	(U.S.)	Bodey *et al* 1986
8.1	(U.S.)	DuPont & Spink 1969
2.85	(U.S.)	Haley *et al* 1985
7.06-12.75	(U.S.)	Kreger *et al* 1980b
3.4	(U.S.)	Scheckler 1978

Of the cases of septicaemia, a large proportion are caused by aerobic or facultative Gram-negative rod-shaped bacteria, and this group of organisms represents from 12% to 37% of blood-borne infections seen in hospitals. When the data for fatality resulting from systemic infections is considered (as summarised in Table 1:2), it can be seen that aerobic and facultative Gram-negative rods are also responsible for a large proportion of deaths, thus reinforcing the position of these organisms as major agents in hospital infections.

The organisms responsible for the majority of cases of nosocomial

septicaemia, as determined from many studies (Armstrong et al 1971; Beytout et al 1987; Brown 1984; Eliasen et al 1986; Eng et al 1987; Finland & Barnes 1978; Forgacs et al 1986; Gatell et al 1988; Ispahani et al 1987; Julander 1987; Lacut et al 1987; Miller & Wenzel 1987; McGowan 1985; 1985; Peltola et al 1987; Rosenthal 1986; Siegman-Igra et al 1988; Whimby et al 1987) are noted below along with the relevent percentage of cases and percentage of total deaths resulting from septicaemia (Table 1:2).

TABLE 1:2. Frequency of Isolation and Fatality for Organisms causing Bacteraemia.

ORGANISM	PERCENTAGE OF INFECTIONS	PERCENTAGE OF TOTAL FATALITIES
GRAM-NEGATIVE:		
E. coli	8.2 - 36.6	8.2 - 19.0
Pseudomonas spp.	3.7 - 18.0	5.1 - 21.4
Klebsiella spp.	3.3 - 28.0	3.8 - 12.7
Enterobacter spp.	0.7 - 7.8	3.1 - 3.8
Serratia spp.	0 - 4.6	2.5 - 4.1
Other aerobic GNB	5.4 - 17.0	5.1 - 10.8
GRAM-POSITIVE:		
Staphylococcus aureus	5.0 - 12.8	0 - 13.9
Coagulase-negative staphylococci	1.2 - 11.5	2.8 - 4.7
Streptococcus pneumoniae	1.3 - 10.7	0.0 - 2.5
Other Streptococci	3.7 - 8.2	2.3 - 7.0

As can be seen in Table 1:2, Gram-negative bacteria, particularly members of the family Enterobacteriaciae, are strongly represented. Escherichia coli, Klebsiella aerogenes, Serratia marsescens, and Enterobacter species, as well as the related non-enteric Pseudomonas

species (especially P. aeruginosa) predominate. Additionally these organisms can be found in mixed infections, and also with anaerobic bacteria and/or Gram-positive bacteria (see above references; Elting et al 1986; Finland & Barnes 1978; Kiani 1979; Miller & Wenzel 1987; Vazquez et al 1987).

In addition to Gram-negative aerobic or facultative organisms, other bacteria are also isolated in cases of septicaemia (see above references), including: Streptococcus species, S. pneumoniae being predominant; Staphylococcus aureus and coagulase-negative staphylococci; Bacteroides species - mainly B. fragilis, and fungi (predominantly Candida species). Of these additional organisms it has been found that Staphylococcus aureus is the most commonly isolated, and although it causes up to 12.8% of bacteraemias, its contribution to fatalities is lower than that of many of the facultative Gram-negative organisms (see table 1:2).

From the data presented in the above table it can be seen that the frequencies of infection and fatalities caused by an organism vary between reports (perhaps reflecting the differing conditions and therapeutic practices found in different hospitals, wards and patient groups - see below) but it can undoubtedly be said that the above mentioned Gram-negative organisms do represent a large proportion of cases of nosocomial septicaemia, and contribute many deaths in hospitals.

Further reports also indicate the increase in prevalence of Gram-negative bacteria in septicaemia (Altemeier et al 1967; DuPont

& Spink 1969; Kreger et al 1980a; McCabe & Jackson 1962a; McCabe & Jackson 1962b; Scully & Henry 1985). It should also be noted that of the Gram-negative rod-shaped bacteria associated with systemic infections, Escherichia coli forms the largest proportion (30 to 70%), and results in a large number of deaths from septicaemia (20 to 60%) (see Table 1:2). Not all strains of E. coli, however, are equally predominant in invasive infections and it is seen that only eight O-serotypes (out of more than 160: see section 1:2) are responsible for more than 50% of E. coli septicaemias (Cheasty et al 1979; Cross et al 1984; Kreger et al 1980a; McCabe et al 1978; Orskov & Orskov 1975). A similar pattern of prevalence of certain O-serotypes can also be seen for other Gram-negative species causing septicaemia, including P. aeruginosa (Dick et al 1988; Moody et al 1972) and Serratia marsescens (Gaston et al 1988). Possible reasons for the prevalence of specific O-serotypes as causes of septicaemia are discussed later in section 1:2.

The presence of bacteria in the blood (bacteraemia) does not necessarily result in the death of an infected individual, as witnessed by the variability in the above mortality rates (see also: Balk et al 1984; van Deventer et al 1988a; Ledingham et al 1988b; McCartney et al 1987). Bacteraemia without clinical indications is observed mainly in individuals who are immunocompetent. Potentially fatal septicaemia, however, occurs in defined groups of immunocompromised individuals as described in section 1:1:2 below. Bacteraemia can proceed to septicaemia, a condition in which clinical signs of infection become apparent. This leads in many cases to a condition called "septic shock" which is the final stage

resulting from serious infection of the blood. Septic shock can also result from a focus of infection in which case the infection is referred to as "sepsis".

A proportion of septicaemias are caused by Gram-positive cocci (see table 1:2), but it has been observed that all of the clinical features and effects found in Gram-negative septicaemia (see section 1:3) are found in many cases of septicaemia caused by Gram-positive organisms (Miller & Wenzel 1987; McCartney et al 1987). Additionally, clinical signs of septicaemia can be observed in complete absence of a positive blood culture (Berger & Beger 1986; Cahill et al 1987; Hass et al 1987; McCartney 1987; MacLean et al 1967).

Analysis of blood has revealed that in many instances endotoxin (which is an integral component of the Gram-negative bacterial envelope and is responsible for many of the effects of Gram-negative septicaemia - see section 1:2 below) is present at high levels during septicaemia in which Gram-negative, Gram-positive, or no organisms have been isolated (Berger et al 1988; Caridis et al 1972; van Deventer et al 1988a; van Deventner et al 1988b; Gaeta et al 1982; Harris et al 1984; Hass et al 1986; Jacob et al 1977; Levin et al 1970; Lumsden et al 1988; McCartney et al 1987; Ohshio et al 1988; Rush et al 1988; Shenep et al 1988; Triger et al 1978). This thus provides a strong indication that endotoxin is the major factor in the pathogenesis of septicaemia. It has, in fact, been determined that the clinical signs of bacterial invasion of the blood only become apparent upon release of endotoxin (see above references).

In cases of Gram-negative septicaemia it has been determined that the levels of endotoxin far exceeds the amount contained on the number of blood-borne organisms present (Caridis _et al_ 1972; van Deventer _et al_ 1988; McCartney _et al_ 1987; Shenep _et al_ 1985a; Shenep _et al_ 1985b; Shenep _et al_ 1988). The presence of endotoxin could result from effective treatment of infection by antimicrobial agents inducing release of endotoxin (Cohen & McConnell 1985; Goto & Nakamura 1980; Shenep _et al_ 1985a; Shenep _et al_ 1985a), thereby possibly accounting for some cases of "non-bacterial" septicaemia, but since the levels are so high - in the range of 5 to 100ng/ml or higher (an organism possesses femtogram amounts of endotoxin and a gross bacteraemia has about 100 organisms per millilitre of blood) - it is highly probable that blood-borne infection results in the release of endotoxin from an endogenous source (see section 1:4). This source may be a septic focus of infection (releasing organisms and endotoxin into the blood), or alternatively the large pool of Gram-negative rods present in the intestine (Caridas _et al_ 1972; Chedid _et al_ 1968; van Deventner _et al_ 1988b; Freeman & Gould 1985a; Jacob _et al_ 1977; Kennedy _et al_ 1965; Sori _et al_ 1988; Tancrede & Andremont 1985). These possible sources would release endotoxin upon an appropriate trigger as discussed in section 1:3. There is much evidence which points to the latter source as being of great significance in septicaemia.

The presence of bacteria in the blood is thus not essential for the development of septic shock, but it is however, beyond doubt that the organisms in the blood are indeed one requirement in the

initiation of the complex processes associated with septicaemia (refer to section 1:3).

Further support for the central role of endotoxin has been obtained by the demonstration that all of the pathophysiological changes associated with septicaemia can be obtained by challenging animals with endotoxin purified from Gram-negative bacteria (see sections 1:3 and 1:4). It can therefore be said that it is the endotoxin of the Gram-negative cell which is of central importance in the development of the clinical syndrome associated with septicaemia. The presence or absence of endotoxaemia thus has an important bearing upon the severity of the syndrome and the survival of patients.

It has, however, been postulated by Galanos and colleagues (Galanos et al 1986) that systemic Gram-negative bacterial infection results in heightened sensitivity of an individual to the activities of endotoxin. It was suggested that any of a number of means may contribute to this "hyper-reactivity", and this seems to support the inter-linked roles for both bacteria and endotoxin in the development of septicaemia.

Gram-negative septicaemia is therefore a result of the invasion of the blood by any of a number of bacterial species, and/or their endotoxins, from either a focus of infection or from an endogenous source, as a result of some form of triggering mechanism, leading to the serious clinical condition referred to as septic shock.

1:1:2. Conditions Predisposing to Gram-negative Septicaemia.

The experience gained in intensive care medicine has revealed a range of conditions which are now known to permit infection and invasion of the blood by potentially pathogenic organisms such as Gram-negative rod-shaped bacteria or their endotoxins. These conditions are diverse and have been well documented (Alford & Hall 1987; Armstrong *et al* 1971; Baumgartner *et al* 1985; Brown 1984; Bryan *et al* 1983; DuPont & Spink 1969; Freeman & McGowan 1978; Gatell *et al* 1988; Harris *et al* 1984; Julander 1987; Klastersky 1985; Kreger *et al* 1980a; Kreger *et al* 1980b; Maki 1981; McCabe & Jackson 1962a; McCabe & Jackson 1962b; McKellar 1985; Parker & Parillo 1983; Sanford 1985; Siegman-Igra *et al* 1988; de la Torre *et al* 1985; Young *et al* 1977). They include surgical manipulation of the respiratory, intestinal and genito-urinary tracts, open-heart surgery, malignancy and its treatment, immunosuppression, immunodeficiency, burns and multiple trauma.

a) Surgical manipulation can permit septicaemia or endotoxaemia (Freeman & Gould 1985a; Nagachinta *et al* 1987; Rocke *et al* 1987). This can result from contamination and infection of wounds and surfaces exposed during surgery from an environmental source. Alternatively, release of endogenous flora from the epidermis or mucous membrane surfaces may occur, thus resulting in direct invasion of the circulation. Manipulation of the intestine is a particular risk for patients as it is a source of many potentially pathogenic facultative Gram-negative organisms, and is also a major source of endotoxin.

b) The lowered immune status present in certain immunodeficiency states and during immunosuppressive treatment as well as that caused

by the therapeutic agents required for treatment of malignancy can also permit invasion of the circulatory system. At particular risk are patients with neutropenia, who very easily become colonised with environmental organisms (Minah _et al_ 1986), and who may possess heightened sensitivity to LPS toxicity (Galanos _et al_ 1986). In addition, deficiencies in other cellular components, and in humoral components of the immune system can lead to successful invasion of the circulation.

c) Severe burns or multiple trauma result in a general lowering of immune status thereby permitting colonisation of wounds. From these wounds, entry into the blood can be gained, where organisms can multiply, release endotoxin, and produce symptoms of septic shock (Deitch _et al_ 1987; Mason _et al_ 1986; Pruitt 1974; Winchurch _et al_ 1987).

d) Instrumentation of patients may also permit the invasion of the blood as a result of colonisation of catheters from an external source, through infusion of contaminated parenteral fluids, or by permitting entry of commensal organisms present on the skin or organisms present in the environment.

e) Intestinal disruption by any of a number of means, permits the release of bacteria or endotoxins into the circulation in larger than normal quantities (see section 1:3). Alteration of the integrity of the intestine could therefore lead to septicaemia or endotoxaemia (van Deventer _et al_ 1988b; Fink _et al_ 1988; Gaffin _et al_ 1981).

f) Impairment of liver function is also a major risk factor for the development of septicaemia or endotoxaemia (Cahill _et al_ 1987; Caridis _et al_ 1972; Gaeta _et al_ 1982; Jacob _et al_ 1977; Lumsden _et_

al 1988; Ohshio et al 1988; Prytz et al 1976; Triger et al 1978; Wardle & Wright 1970). This occurs because of the role of the liver as a major organ of removal of bacteria and endotoxin in healthy individuals (see section 1:3). Thus alteration of the efficacy of this function would permit entry and persistence of bacteria and bacterial products in the circulation.

Further groups at high risk include children up to one year old, especially premature neonates (see for example Hill 1985) and aged people (both groups having a lowered immune status) who often succumb to septicaemia and meningitis caused by enterobacteria - mainly E. coli and Klebsiella pneumoniae.

Finally, a recent addition to conditions resulting in septicaemia has become noticeable - it has been observed that people with the acquired immune deficiency syndrome (AIDS) are susceptible to septicaemia and this has become a recognised cause of death among AIDS patients (Celum et al 1987; De Wit et al 1988; Eng et al 1987; Fischl et al 1986; Nadelman et al 1985; Sperber & Schleupner 1987). Septicaemia caused by species of Salmonella are to be found more often than in the other groups of patients mentioned above. This perhaps reflects the particular mode of immunosuppression found in AIDS patients and also a requirement for a different mode of pathogenesis for the organisms.

All of the above factors can therefore be of importance in the progression of septicaemia and the definition of Gram-negative septicaemia described above can now be expanded. Septicaemia can now

be said to represent a nosocomial infection in patients who are immunocompromised, resulting from invasion of the blood by Gram-negative organisms - particularly enterobacteria - or endotoxin from a site of infection, from an endogenous source, or from an exogenous source.

Despite the prevalence of Gram-negative bacteria as causes of nosocomial septicaemia, many cases of septicaemia can be seen to be the result of infection outside the hospital environment (Bisbe et al 1988; Finland & Barnes 1978; Ispahani et al 1987; McCabe & Jackson 1962a; Scheckler 1978; Siegman-Igra et al 1988; de la Torre 1985). These cases nevertheless result in hospitalisation of individuals and a similar clinical outcome. Community-acquired infections therefore add to the magnitude of the problem arising from systemic infections caused by Gram-negative bacteria.

It has been observed that the species commonly causing community-acquired septicaemia differ slightly in proportion from those causing nosocomial septicaemia, with E. coli and Klebsiella pneumoniae forming a greater proportion of cases (see above references).

Another important factor to note is that the severity of the disease underlying the septicaemia has a bearing on the outcome of infection (Ispahani et al 1987; Kreger et al 1980b; Maki 1981; Miller & Wenzel 1987; McCabe & Jackson 1962a; McCabe & Jackson 1962b). It has been demonstrated that more-severely compromised individuals (that is, those with an underlying condition which is itself fatal) have a

lower chance of survival from septicaemia than do less compromised people. In addition, those people acquiring septicaemia within the community possess similar modes of reduction in immune competence to patients acquiring nosocomial infection, but their compromised states are generally less severe, and patients acquiring septicaeia within the community show higher rates of recovery.

It is obvious, therefore, that certain groups of individuals are particularly susceptible to this type of infection. In these groups there is a requirement for some form of immunodeficiency, the severity of which has a bearing upon the outcome of infection. The range of conditions which may enable opportunistic infection and lead to septicaemia by any of a range of potentially pathogenic Gram-negative bacteria is, however, large.

The host factors responsible for protection against septic shock resulting from Gram-negative septicaemia, and treatment and prevention strategies will be discussed in later sections (1:3 and 1:5 respectively).

1:2. Structure and Function of Lipopolysaccharide in Gram-negative Bacteria.

1:2:1. Structure of Gram-Negative Bacteria.

The Gram-negative cell has a more complex structure than that seen in Gram-positive bacteria. The surface components of Gram-negative bacteria comprise three layers as represented diagrammatically in figure 1:1.

The outer membrane (OM) of Gram-negative bacteria is a unique structure and is generally regarded as the outermost component of the cell. In many cases, however, an additional polysaccharide component is present on the surface. These "capsules" or "slimes" are important virulence factors for many strains of Gram-negative bacteria which cause septicaemia (see below), but are by no means essential for the development of septic shock.

Beneath the outer membrane is the periplasmic space which contains many proteins involved in bacterial metabolism, and additionally lipoproteins which link outer membrane covalently to a thin layer of peptidoglycan. Finally, below the peptidoglycan is the cell membrane of the bacterium.

It is the lipopolysaccharide (LPS) of the OM that is responsible for causing the symptoms associated with septicaemia, but before discussing the toxic activities of LPS, the structure and composition of this molecule and its role in virulence will be summarised.

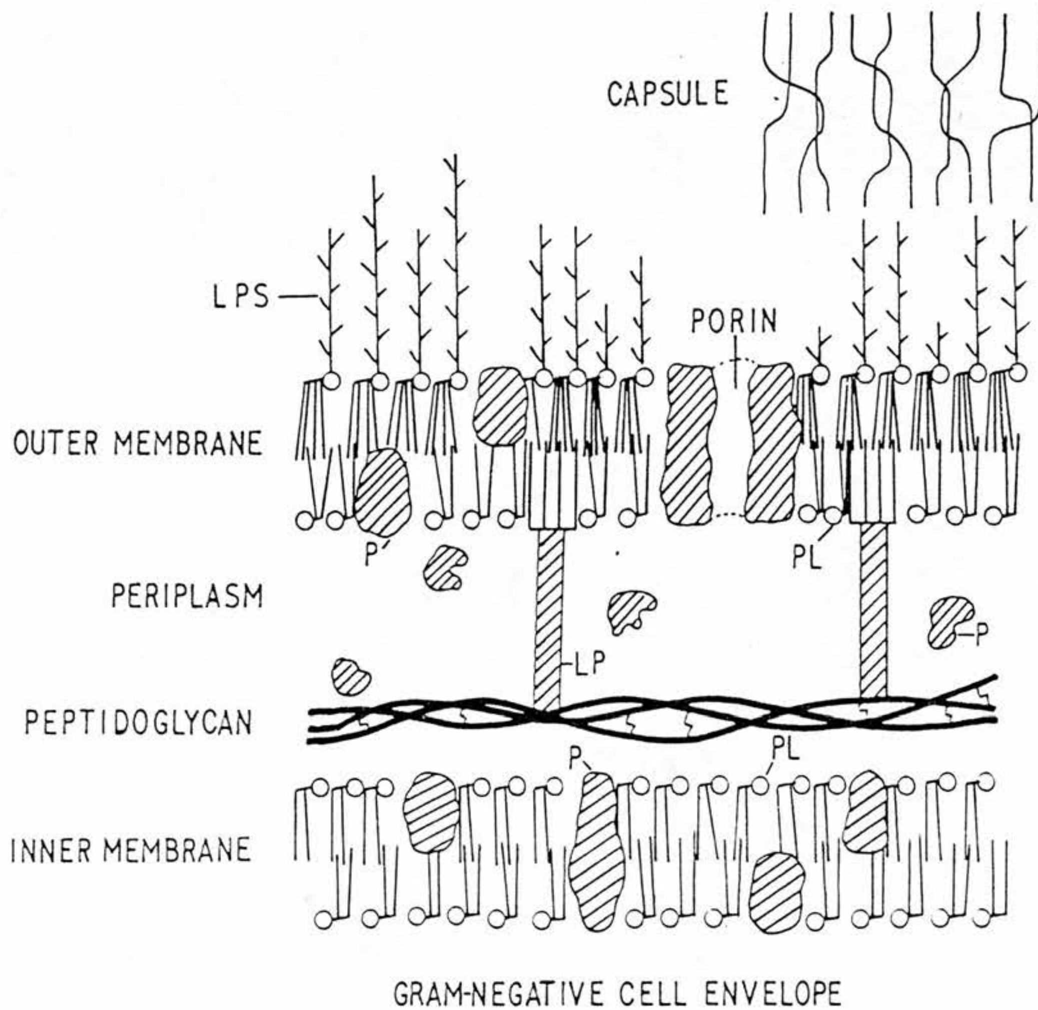


Figure 1:1. Schematic diagram of the Surface Components of Gram-negative Bacteria.

(from "Bacterial Cell Surface Techniques", Hancock and Poxton, John Wiley & Sons, Chichster, 1988).

1:2:2. Structure of the Outer Membrane (OM) of Gram-negative Bacteria.

The OM of Gram-negative bacteria is a complex structure composed of phospholipids, proteins and LPS. Of the constituents of the outer membrane, LPS is the major component, representing more than 50% of the weight of extracted OM (Costerton *et al* 1974). Additionally, lipopolysaccharide is the most antigenic structure of the outer membrane and also the most structurally diverse.

The lipid component of the lipopolysaccharide molecule (lipid A) interacts hydrophobically with phospholipids which form the inner leaflet of the bilamellar outer-membrane, and the polysaccharide component of LPS extends outwards from the cell surface. In addition to LPS, the OM also possesses protein components, the majority of which represent trans-membrane pores. As well as these structures, the OM may be traversed by tubular protein polymers which can be structures required for motility (flagella) or for attachment (fimbriae).

The outer membrane of Gram-negative bacteria is therefore a highly complex structure, of which the lipopolysaccharide component is intimately involved in the processes of septicaemia.

1:2:3. Structure of Lipopolysaccharide.

Lipopolysaccharide is a complex amphipathic molecule which possesses three distinct regions: i) a polysaccharide composed of repeating oligosaccharide units - the O-antigen; ii) an oligosaccharide "core"

region; and iii) an inner lipid component - Lipid A.

O-polysaccharides.

The O-polysaccharide or, as it is more usually called, the O-antigen, represents the outermost region of LPS (see figure 1:2), and has been found to be composed of one to many repeating oligosaccharide units commonly containing from 2 to 5 sugars. Larger O-antigen units can, however, be found. The oligosaccharides of these units form either a linear or branched structure (Hitchcock et al 1986; Luderitz et al 1984; Orskov et al 1977; Rietschel & Brade 1987; Rietschel et al 1984b; Westphal et al 1983). These oligosaccharide units form a repeating structure which extends outwards from the cell surface in a helix. The repeating units are hetero-oligosaccharides (composed of several different sugars) in most cases, but a few examples of homo-oligosaccharides (repeating units comprising one sugar) have been found in, for example, Klebsiella and Enterobacter species.

O-antigens have been found to contain a wide range of sugar molecules including hexoses, hexosamines, deoxyhexoses, dideoxyhexoses, deoxyhexosamines, pentoses, and uronic acids (Knirel et al 1988; Orskov et al 1977; Westphal et al 1983; Wilkinson et al 1973; Wilkinson et al 1975; Wilkinson 1977). In addition, non-sugar constituents including phosphoryl, glyceryl, acetyl, pyruvyl, and ethanolaminyl groups may be present.

As a result of the wide range of possible constituents, it can be said that the O-antigen is a chemically and antigenically highly

diverse component of LPS and therfore of the Gram-negative bacterial cell surface. This is reflected in the ability to classify strains of Gram-negative organisms by means of their O-antigen, which results in a number of distinct "O-serogroups" that possess strain-specific polysaccharide structures. For example, E. coli has been shown to have over 160 O-serogroups. The antisera to some of these O-antigens cross-react with other O-antigens to a greater or lesser degree, but each O-antigen can be shown to possess a distinct structure. Additionally, for other Gram-negative species an O-antigen-dependent typing system has been obtained. These include Pseudomonas aeruginosa (Lui et al 1983); Klebsiella aerogenes, (Kauffmann 1969); Salmonella, (Edwards & Ewing 1972; Kaufmann 1969); and Serratia marsescens (Guinee et al 1987; Pitt & Erdman 1984).

In addition to cross-reactivity observed within a species or genus, some cross-reactivity can be seen between genera (Orskov et al 1977; Perez-Perez et al 1986). It is known that certain E. coli O-antigens show cross-reactivity with some strains of Vibrio cholerae, Salmonella spp. and Shigella spp. reflecting the clinical syndromes with which these serotypes are associated (see section 1:2:3). Other such cross-reactivities are also known to occur. Thus despite the diversity of components of O-antigen, some structural and antigenic similarities can be observed between some serotypes of certain organisms.

The structurally variable O-antigen component of lipopolysaccharide is covalently linked to the "core" oligosaccharide.

Core oligosaccharides.

Core oligosaccharide acts as a bridge between the O-antigen and the lipid A. O-antigen is bound through its proximal saccharide to the terminal or sub-terminal sugar in the core (usually a glucose molecule), with lipid A binding at the opposite end of the core from the O-antigen - figure 1:2.

The core region (which together with lipid A forms the core glycolipid or CGL) of LPS is a short oligosaccharide composed of usually 11 monosaccharides (Jansson et al 1981; Orskov et al 1978). Within the core itself there are two distinct regions - the inner and outer cores. Compositional, structural, and immunochemical analyses of core oligosaccharides from several different genera and species of Gram-negative bacteria have been carried out, showing that many similarities exist, especially within the family Enterobacteriaciae.

Analysis of core oligosaccharide structure has been facilitated through the development of a series of "rough"-mutant strains from various organisms (Brade et al 1988; Orskov et al 1977; Westphal et al 1983). Mutants have been obtained which possess progressively shorter oligosaccharides substituted onto lipid A. The mutants are designated as the Ra, Rb, Rc, Rd, and Re chemotypes. The Ra chemotype possesses a complete core oligosaccharide, Rb has one sugar less, and so on until the Re chemotype which possess only one type of sugar attached to lipid A - see figure 1:3. Re-CGL is the smallest obtainable LPS produced "naturally" through mutation - no strains which possess only lipid A are found.

FIGURE 1:2. Schematic Representation of Gram-negative Bacterial Lipopolysaccharide.

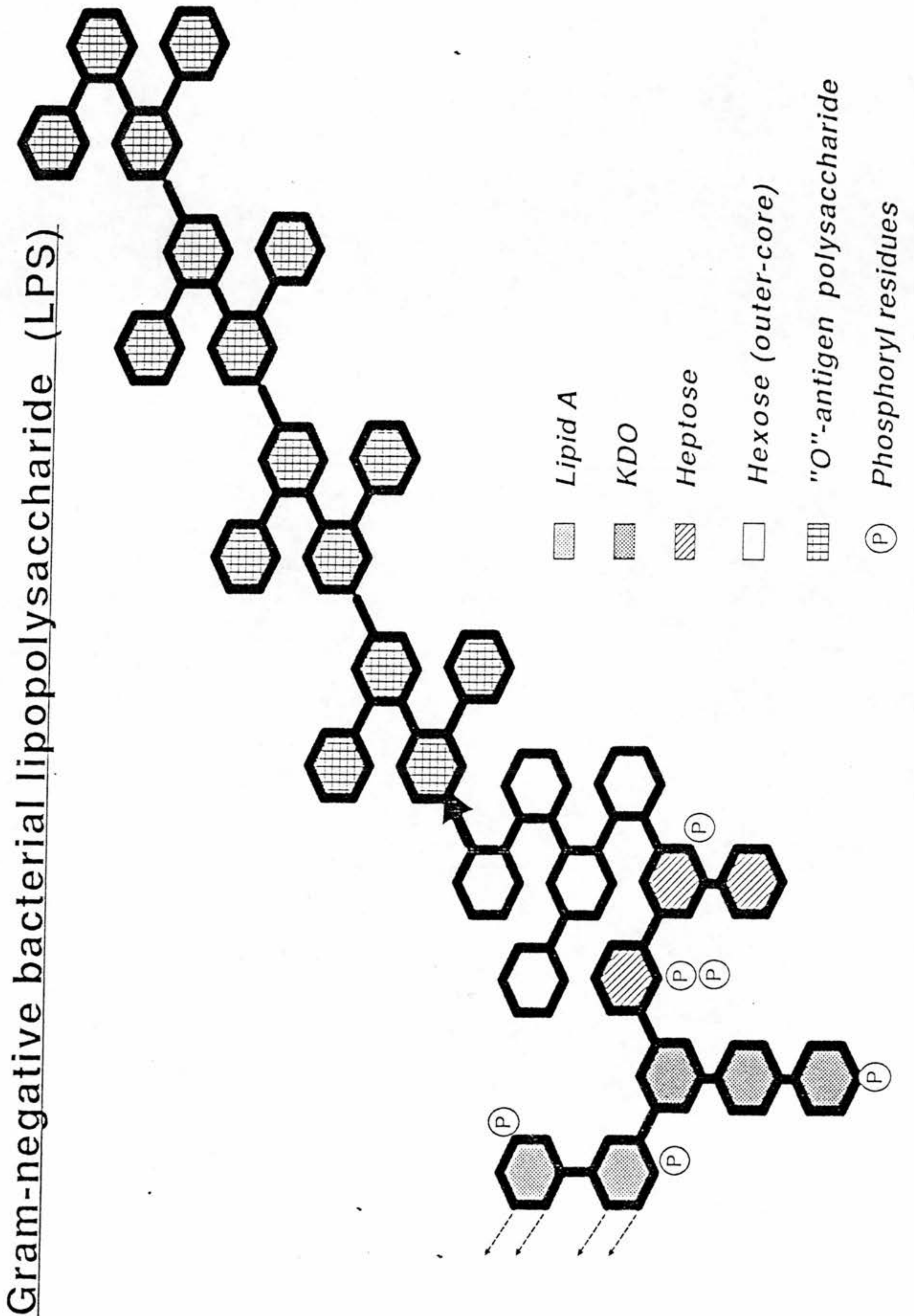
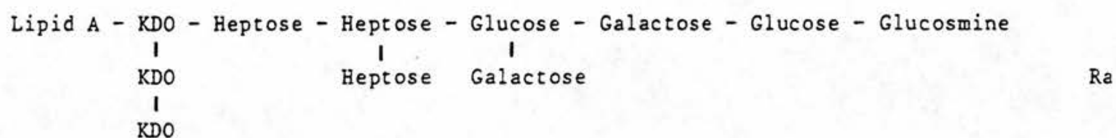
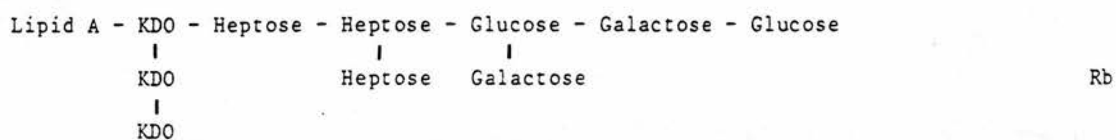
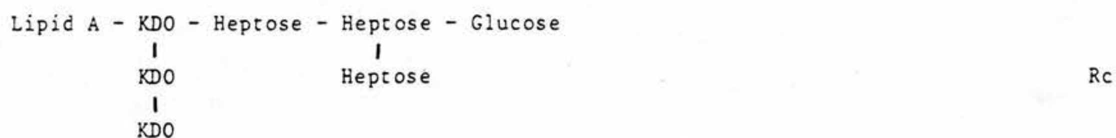


FIGURE 1:3. Rough Lipopolysaccharide Chemotypes of Salmonella.







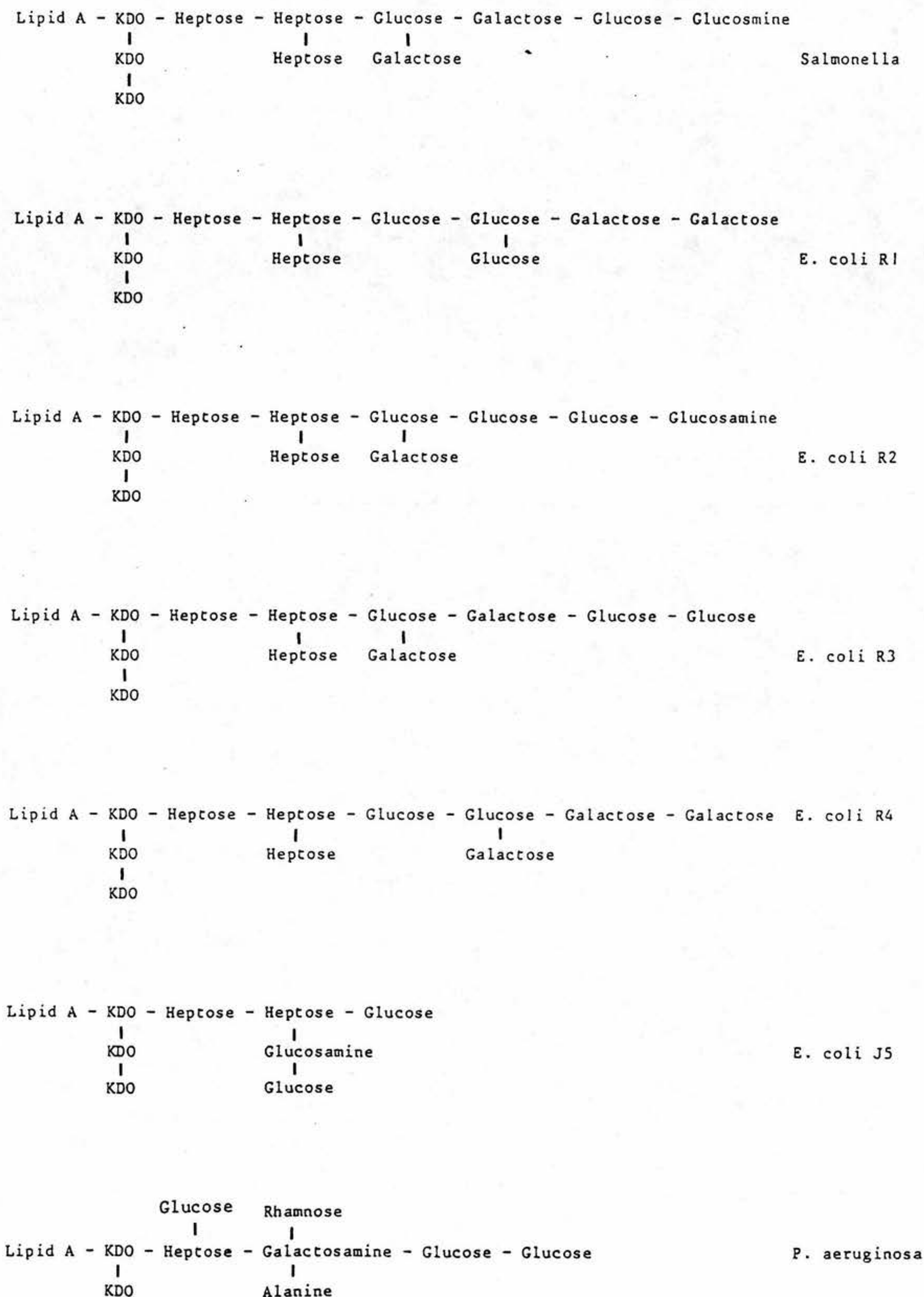
Rough mutants of S. minnesota, S. typhimurium, Escherichia coli, and Pseudomonas aeruginosa have been produced, and additionally other organisms have been studied (Holme et al 1968; Hudson et al 1978; Luderitz et al 1966). Those organisms of particular significance in Gram-negative septicaemia whose core structures have been well-studied are E. coli and P. aeruginosa, but little is known of the structures of the cores of other Enterobacteriaciae causing septicaemia.

It was originally believed that core oligosaccharides from many different species of enterobacteria possessed an identical structure, but it has subsequently been determined that E. coli itself possesses five different core structures and the genus Salmonella possesses only one. Most of the variation which has been observed occurs in the outer core region.

The sugars which are the common components of outer core are glucose, galactose, and glucosamine. These can be found in all enterobacterial cores so far examined, but they have been shown to be present in different structural conformations - see figure 1:4 (Eskenazy et al 1977; Jansson et al 1981; de Jongh-Leuvenink et al 1985; Luderitz et al 1982; Peters et al 1985; Perez-Perez et al 1986; Schimdt et al 1970; Tsang et al 1987). Additionally, outer cores may possess non-sugar components of which phosphate and acetyl groups are predominant.

In contrast to the outer core, the inner region shows a greater

FIGURE 1:4. Structures of Lipopolysaccharide Core regions of Salmonella, E. coli, and P. aeruginosa.



degree of structural conservation between species. This region comprises three molecules each of glycero-manno-heptose and keto-deoxy-octulosonic acid (KDO), as well as phosphate, diphosphate, and ethanolamine groups - figure 1:4 (above references; Brade et al 1986; Brade et al 1988; Tacken et al 1986). This region therefore shows a greater degree of structural conservation between species and indeed is identical among all Enterobacteriaciae.

Study of the core of Pseudomonas aeruginosa (Fensom & Meadow 1970; Koval & Meadow 1975; Koval & Meadow 1977; Kropinski et al 1979; Rowe & Meadow 1983; Sawada et al 1985; Wells et al 1985) has revealed a similar structure to that of enterobacterial cores. Differences are, though, observed in the outer core where glucose and galactosamine are present with the addition of a deoxyhexose (rhamnose) and an amino acid (alanine) - figure 1:4. The inner core does, however, seem to possess greater similarity to that of E. coli and Salmonella, though only two KDO molecules and a single heptose appear to be present. The gross structure and composition of this core is, though similar to that of enterobacteria.

Conservation of core structure is reflected in the cross-reactivity of antibodies (monoclonal and polyclonal) to this region across many genera (Bogard et al 1984; Bogard et al 1987; Brade & Galanos 1983; de Jongh-Leuvenink et al 1986; Kirkland et al 1986; McCallus & Norcross 1987; Nelles & Niswander 1984; Pollack et al 1987; Siber et al 1985; Sidberry et al 1985; Young et al 1975a).

The core region of LPS is therefore a chemically and structurally

highly conserved region in comparison to the O-antigen, though minor differences do exist.

As mentioned above, it is this core region which forms the link between the O-antigen and the membrane-embedded lipid A component of lipopolysaccharide.

Lipid A.

Lipid A is the innermost component of LPS and is embedded within the outer leaflet of the OM through lipophilic interactions with phospholipids. Lipid A is bound to the core region of LPS through one of the KDO molecules of the inner core.

This hydrophobic lipid A moiety itself has a complex structure (Batley *et al* 1984; Batley *et al* 1985a; Batley *et al* 1985b; Batley *et al* 1985c; Brade *et al* 1988; Burton & Carter 1984; Luderitz *et al* 1973; Luderitz *et al* 1984; Mattsby-Baltzer & Alving 1984a; Mattsby-Baltzer *et al* 1984a; Mattsby-Baltzer & Alving 1984b; Qureshi *et al* 1985; Raetz 1984; Rietschel *et al* 1984a; Westphal *et al* 1983). It is composed of a di-glucosaminyl-glucosamine backbone to which fatty acid chains, phosphates, and core are bonded - see figure 1:5. The major structural differences found between lipid A from different bacteria are in the composition of fatty acids and also in the nature of the substituents present on the phosphate units. Components which have been shown to be common substituents are phosphorylethanolamine, **D**-glucosamine, phosphate, furanosidic-**D**-arabinose, and 4-amino-4-deoxy-**L**-arabinose.

Lipid A is therefore the least variable component of LPS, showing a high degree of structural conservation between genera (Drewry et al 1973; Galanos et al 1984; Homma et al 1985; Matsuura et al 1985; Mattsby-Baltzer et al 1984b; Westphal 1983). In addition, lipid A from different organisms show immunochemical similarities (Brade & Brade 1985; Brade et al 1986; Elkins & Metcalf 1985; Galanos et al 1984a; Kasai et al 1985; Kirkland et al 1985; Mutharia et al 1984; Pollack et al 1987; Ramachandra et al 1988; Rietschel et al 1987).

It has been determined that the toxic activities (section 1:3) of LPS reside in the lipid A component, and the continuing advances of knowledge of the structure of lipid A is enabling determination of structural components responsible for toxicity and immunogenicity (Arata et al 1988; Chaby et al 1987; Elkins & Metcalf 1985; Kanegasaki et al 1984; Kasai et al 1985; Kotani et al 1985; Kumazana et al 1988; Proctor & Textor 1985; Rietschel et al 1984b; Shiba et al 1984; Shimizu et al 1988; Takada et al 1985; Takahashi et al 1987; Takayama et al 1984a; Takayama et al 1984b; Tanamoto et al 1984) The toxic activities of lipid A and LPS (which are also conserved between genera) are discussed in section 1:3.

The lipopolysaccharides of Gram-negative bacteria associated with septicaemia thus show many structural, immunochemical, and toxic similarities between species. Variation does, however exist, and can be seen in each of the three components of the lipopolysaccharide molecule. Greater variation is observed between enterobacterial LPS and those of Bacteroides species, which is reflected in both

immunogenic and toxic differences (Hofstad 1988; Johne _et al_ 1987; Luderitz _et al_ 1984; Luderitz _et al_ 1987; Rietschel _et al_ 1987). Other organisms which are less closely associated with septicaemia show even greater divergence of structure of LPS components, although the basic gross structure remains (see for example Hitchcock _et al_ 1986; McCartney & Wardle 1985). Despite this, lipopolysaccharide is a highly important component of an organism, and plays an important role in the pathogenesis of septicaemia.

1:2:4. Biosynthesis of Lipopolysaccharide and Effect of Growth Conditions.

The mechanisms involved in the synthesis of the O-antigen, core and lipid A components of lipopolysaccharide have been determined at both biochemical and genetic levels (Brahmbhatt _et al_ 1988; Ishiguro _et al_ 1986; Orskov _et al_ 1977; Osborn _et al_ 1972; Wilkinson 1977).

Steps involved in the synthesis of lipid A continue to be uncovered (Anderson _et al_ 1985; Coleman & Raetz 1988), as the structural requirements for toxicity and immunogenicity become clearer.

The core-glycolipid component is formed in a stepwise process at the cell membrane by addition of individual core sugar units onto lipid A (Goldman _et al_ 1988a; Goldman _et al_ 1988b; Orskov _et al_ 1977; Wilkinson 1977). O-antigen oligosaccharide units which have been synthesised step-wise onto a carrier lipid, are then polymerised onto core-glycolipid. The complete LPS molecule can then be transferred to the outer surface of the outer membrane.

Variation in the number of O-antigen units substituted onto core-glycolipid can be seen between organisms (Chester & Meadow 1975; Gaston et al 1988; Goldman & Leive 1980; Hitchcock et al 1986; Palva & Makela 1980; Peterson & McGroarty 1985; Rivera et al 1988). Even within a culture of a particular strain of an organism, heterogeneity of LPS chain length is observed, although a predominant length of chain is produced. In addition, substitution with a single O-antigen unit (S-R LPS) and no O-antigen units (R-LPS) are present in populations of bacteria.

It has been determined that alteration of the growth conditions of a bacterium results in alteration of LPS chain length and, in some cases, sugar composition (Chester & Meadow 1975; Collins 1964; Dodds et al 1987a; Dodds et al 1987b; ; Hraback et al 1981; Kropinski et al 1987; Ombaka et al 1983). When growing rapidly in a rich medium LPS is predominantly of shorter chain or S-R type. As a response to progressively less conducive growth conditions, the chain length of LPS is observed to increase, often in conjunction with a decrease in growth rate. Similar alteration in O-antigen chain length has been observed in vivo for P. aeruginosa in infections in cystic fibrosis (Cochrane et al 1988). This has an important bearing in vivo as the presence of LPS with greater numbers of O-antigen units has been shown to confer resistance to serum bactericidal activity (see section 1:2:3).

The alteration of LPS has also been observed over the growth curve, and has been shown to affect virulence and toxicity, as well as resistance to antimicrobial agents and to host defence mechanisms

(Benjamin *et al* 1986; Finch & Brown 1975; Finch & Brown 1978; McCallus & Norcross 1987; Russell & Furr 1987; Russell *et al* 1987; Shearer & Legakis 1985; Weiss *et al* 1986).

The growth conditions of a Gram-negative organism thus have an important influence upon the structure of the LPS of the outer cell membrane and therefore upon the virulence and toxic activities of the molecule (see sections 1:2:3 and 1:3:1 respectively).

1:2:5. Role of Lipopolysaccharide in Virulence.

Since lipopolysaccharide is a major surface component it is expected that it must perform some role in the virulence of an organism, besides its role as the highly active endotoxin.

As mentioned above, lipopolysaccharide forms the major fraction of the Gram-negative bacterial outer membrane, and as such acts as a selectively permeable barrier between the cell and its environment. This, in itself, is not a determinant of virulence, but it is an invaluable role for LPS (and OM) in cell integrity and viability.

As a result of the many negatively-charged groups present throughout the length of the LPS molecule (see section 1:2:2), positively charged ions and molecules may be sequestered from the environment onto the LPS at the cell surface. These ions could then be taken up and used for essential processes in cellular metabolism. This sequestration of positive ions may provide a distinct advantage when these cations are in limited supply as may be observed when cells are growing *in vivo*.

Lipopolysaccharide plays a very important role in the process of invasion from local sites into the bloodstream. E. coli, the commonest organism causing septicaemia, has over 160 O-serotypes (and hence over 160 different LPS structures), but only a small fraction of these have been found to result in septicaemia (or other invasive infections) (Orskov 1978; Orskov et al 1977). A similar limitation of O-antigen distribution has been determined for other clinical conditions caused by E. coli (see table 1:3).

Table 1:3. Relationship of O-serotype to Clinical Condition for E. coli.

CLINICAL CONDITION	ASSOCIATED O-SEROTYPES
septicaemia	O1;O2;O4;O6;O7;O8;O9; O11;O18;O22;O25;O75.
urinary tract infection	O1;O2;O4;O6;O7;O8;O9; O11;O22;O25;O62;O75.
neonatal meningitis	O1;O6;O7;O16;O18;O83.
healthy faeces	O1;O2;O4;O6;O7;O8; O18;O25;O45;O75;O81.

As can be seen in the above table, there are only 12 O-serotypes of E. coli commonly found in cases of septicaemia. The O-serotypes responsible for particular types of infection have been shown to possess very similar structures thereby confirming the role of LPS as an important determinant in the pathogenic processes of many infections.

A similar situation to that seen for E. coli may be observed with other Gram-negative organisms, but less is known of the O-antigenic structure of many of these, therefore little can be concluded,

although it is observed that a limited range of O-antigen serotypes of Serratia marsescens causes the majority of systemic infections (Gaston et al 1988). One exception to this is Pseudomonas aeruginosa, in which all O-serotypes can be found causing septicaemia (and other infections), but those isolated belong predominantly to only a small number of the O-serotypes (Dick et al 1988; Moody et al 1972; Zweerink et al 1988b). It is possible, therefore, that the situation where all serotypes can cause infection but some are more predominant, may be observed more commonly for non-commensal organisms which cause septicaemia, while commensal organisms show a limited range of O-serotypes which can produce invasive infections.

It has been determined that LPS may effect invasion of the circulation by aiding adherence of bacteria to tissues and transfer across the tissue barrier. It appears that the O-serotypes of E. coli which enable invasion may have a role in augmenting adherence to host cell surfaces. This is best exemplified for intestinal infections caused by Shigella, Salmonella, and some strains of E. coli (Nevola et al 1985; Nevola et al 1987; Smith 1977; Smith & Parsell 1974). These strains possess acidic O-polysaccharides which in some way permit local invasion of tissues. It is most likely, therefore, that bacteraemia arising from endogenous flora where perforation of colonised surfaces has not occurred may therefore result through LPS of particular structure and composition enabling adherence of bacteria to mucous membranes followed by entry into blood.

Once entry into the circulation is achieved, a bacterium then faces the immunological defences of the host, and here again LPS plays an important role in the prevention of an effective immunological response to the bacterial cells. There are several means by which this may be acheived:

i) One possibility by which an organism can prevent an immune response being mounted is by mimicking a host antigen. Because of the wide range of sugar components of lipopolysaccharides it is possible that some O-antigens may resemble certain host polysaccharides, but this does not appear to be a common occurrence for LPS, as the vast majority of O-antigens are highly immunogenic and efficiently elicit production of specific antibodies (see section 1:4). One example of known cross-reactivity exists between the O-antigen of _E. coli_ O86 and blood group B antigen (Springer 1971).

ii) It has been shown that some lipopolysaccharide O-antigens possess anti-phagocytic and/or anti-complement activity (Brown & Williams 1985; Williams _et al_ 1983; Williams _et al_ 1986; Young 1972; Young 1975b), thereby preventing removal of bacteria from the circulation and/or bacteriolysis by serum. This has been demonstrated by the use of rough mutants (lacking O-antigen) of virulent strains of bacteria which are phagocytosed or lysed by complement far more rapidly than their O-antigen containing parent strains (Betz _et al_ 1981; Makela _et al_ 1973; Orskov 1978; Porat _et al_ 1987; Sansano _et al_ 1985; Schiller 1988; Shaio & Rowland 1985) in the absence of specific antibodies. Similar results have also been obtained by comparison of serum sensitive and serum resistant O-antigen containing strains of organisms (Ciurana & Tomas 1987;

Cryz *et al* 1984; DeMatteo *et al* 1981; Goldman & Lieve 1984; Goldman *et al* 1984; Grossman *et al* 1987; Jessop & Lambert 1986; Loos & Clas 1987; Michael & Landy 1961; Sansano *et al* 1985; Tomas *et al* 1986; Tomas *et al* 1988; Taylor 1983). These serum resistant strains often possess LPS with greater substitution of O-antigen units onto core glycolipid, but the sugar composition and structure of the molecule may also have a bearing (Jiminez-Lucho *et al* 1987; Rozenberg-Arska *et al* 1986).

It appears that lipopolysaccharide activates complement at a site distant from the cell surface and thus lysis is prevented. Further evidence for the role of LPS as an anti-complement component has been demonstrated through the ability of free LPS to prevent killing and phagocytosis (Tanamoto *et al* 1984; Vukajlovich 1986; Young 1975), probably by activation of complement therefore resulting in its depletion.

Lipopolysaccharide therefore has a very important role in prevention of complement dependent processes, through any of a number of means, but the precise requirements for these antiphagocytic and anti-complementary activities are uncertain.

iii) It has been postulated in several reports that the O-antigen prevents access of antibodies to antigens on the surface of the OM (Bentley & Klebba 1988; Jessop & Lambert 1985; Kelly *et al* 1987; Saxen *et al* 1986; Shenep *et al* 1987; Vuopio-Varkila *et al* 1988a). This would therefore prevent access to conserved structures of the OM such as proteins and the core region of LPS, which may otherwise be protective. This factor could represent another means of preventing an effective immune response.

iv) LPS is a polyclonal B-cell mitogen (see section 1:3) and

stimulates the production of many antibodies which do not recognise antigens on the Gram-negative bacterial cell surface. This could serve to divert a specific immune response away from the bacterium or endotoxin, thereby lowering the efficiency of the anti-bacterial and anti-LPS response.

v) The major means of protection from bacterial pathogens is by the production of specific antibodies (see section 1:4). The endotoxin molecule , particularly the O-antigen, is highly immunogenic and elicits the production of many antibodies to each of the three regions. These antibodies may possess opsonic, bacteriolytic, or anti-endotoxic activity. Despite the advantage of possession of antibodies, this does not ensure their effective activity. In some cases antibody has been shown to bind to O-antigen at its distal position, thereby permitting activation of complement at a site distant from the bacterial outer membrane thus preventing bacteriolysis (Engels *et al* 1985; Rozenberg-Arska *et al* 1986). Antibodies to conserved regions of LPS and also proteins could possibly permit bacteriolysis by complement, but the O-antigen prevents access of antibodies to these sites by steric hindrance (see *iii*). The inhibition of binding to conserved sites on the outer-membrane may present less of a problem *in vivo* as endotoxin produces its toxic actions only upon liberation from the cell (section 1:3), which results in exposure of conserved regions and would permit binding of antibodies.

vi) Lipopolysaccharide also has a major role in the alteration of many host functions of the immune and other systems. This function of LPS in pathogenesis is discussed in detail in section 1:3.

There are thus many possible means by which lipopolysaccharide can influence the virulence of Gram-negative bacteria with regard to systemic infection, but its most clearly defined role is that as endotoxin, which results in many of the pathophysiological alterations seen in septicaemia as described in section 1:3.

1:2:6. Other Bacterial Components Affecting Virulence in Septicaemia.

There are many components of the bacterial cell in addition to lipopolysaccharide, which may have an important bearing on the pathogenic processes involved in septicaemia. These factors may be either components of the outer membrane or extracellular molecules.

Surface Components.

All components of the surface of Gram-negative bacteria appear to play a role in the pathogenesis of septicaemia, among those are outer membrane proteins, fimbriae and capsule (see for example Brubaker 1985).

Outer-membrane proteins (omp) play a vital role in the ability of a cell to assimilate nutrients from the environment, and it has been shown that alteration of growth conditions results in changes in expression of omp. As with LPS, variation observed under different conditions may also have an influence on pathogenicity, but the precise role of alteration in omp is not clearly defined in septicaemia.

Flagella are major components of the envelope of many Gram-negative bacteria, and represent an antigenic structure which is used for

serotyping purposes. These flagella are responsible for motility of Gram-negative bacteria, but they appear to possess little role in the pathogenesis of septicaemia although they may be important in other infections.

Fimbriae present on the cell surface also appear to be an important factor during septicaemia. There are several types of these protein-polymers, which all serve to aid in attachment to, and invasion of host surfaces. Recent work by Saukkonen et al (1988) has determined a putative role for fimbriae during invasive infections, and particularly in septicaemia, by aiding adherence of cells to vascular endothelium therefore preventing the removal of bacteria by phagocytosis. This could also permit local tissue damage (Steadman et al 1988) and contribute to the septic syndrome by causing release of mediators from the endothelial tissue and the cellular immune system at the sites of bacterial adherence.

Of greatest importance to invasive infections, secondary to that of LPS, is the capsule of an organism. This is a polysaccharide component which is present in many strains of Gram-negative bacteria as the outer-most structure external to the outer-membrane.

The capsular polysaccharide can vary from a thin layer on the bacterial surface, to a massive gelatinous excretion which is visible under the light microscope with appropriate staining. The components of capsules are as diverse as those found in lipopolysaccharide, with many saccharide and non-saccharide units

present (Jann & Jann 1977; Jann & Jann 1983; Jann & Jann 1987; Orskov et al 1977; Sutherland 1985). It has indeed been determined that some capsules represent extracellular O-antigen and are therefore structurally and antigenically identical to the polysaccharide of LPS.

Certain capsules enable invasion of tissues and blood, and also perform anti-phagocytic and anti-complementary functions (Allen et al 1987a; Allen et al 1987b; Bortolussi et al 1979; Cross et al 1984; Cross et al 1986; Stevens et al 1980; Tomas et al 1986; Welch et al 1979; Williams et al 1983; Williams et al 1986). These activities are performed as a result of the high negative charge and high hydrophilicity of the capsule. If, however, anti-capsular antibodies are present then activation of complement and antibody-dependent phagocytosis can occur, resulting in cell lysis and/or removal (Bortolussi & Ferrieri 1980; Cross et al 1983; Raff et al 1988; Williams et al 1988). This is overcome in some strains through production of a non-immunogenic capsule, such as the K1 or K5 capsule of E. coli, or by production of a mass of capsular material. Strains carrying the K1 or K5 capsules are, in fact, observed to cause a large proportion of invasive infections (Cheasty et al 1978; Cross et al 1984).

Despite the advantages for a pathogen to possess a capsule, many non-capsulate organisms can also effect invasion and resist phagocytosis or complement lysis as a result of the presence of certain lipopolysaccharide structures. Capsules are particularly important for strains which would otherwise be serum sensitive, as

observed for some invasive strains of *E. coli* which possess LPS which does not confer resistance to serum killing or phagocytosis. Additionally, capsule is also important to pathogenic organisms which naturally possess only rough LPS on their surfaces as witnessed by the presence of rough strains of *E. coli* in invasive infections (Cheasty *et al* 1978; Cross *et al* 1984) and *P. aeruginosa* in some localised infections (Cochrane *et al* 1988; Fomsgaard *et al* 1988; Kelly *et al* 1987). In addition, organisms which possess only rough type LPS, or lipo-oligosaccharide, such as *Neisseria meningitidis* and *Haemophilus influenzae*, capsule assumes great significance in pathogenesis.

It has been observed that, as with LPS, growth conditions markedly alter expression of capsular polysaccharides. At high growth rates very little capsule is produced, but during nutrient limitation there is a massive increase in production of capsule and concomitantly surface hydrophilicity, and therefore increased virulence.

Extracellular Components.

Many Gram-negative organisms produce extracellular proteins (see for example Brubaker 1985). The production of these toxins or enzymes could therefore contribute to the pathogenic and pathophysiological processes of septicaemia, as well as in other disease processes. In septicaemia, no clearly defined role has been determined for toxins from most organisms, except *P. aeruginosa* for which exotoxin A is believed to be an important determinant (Cryz *et al* 1984; Miller & Wenzel 1987; Pollack 1984; Pollack & Young 1979; Pollack *et al*

1983). The putative role of these molecules must therefore not be ignored.

Many components of the Gram-negative organism, therefore, may have a part to play in the pathogenesis of septicaemia, but those most closely linked to the development of invasive infections are LPS and capsule. Of these, LPS is undoubtedly by far the more important factor, as discussed in this and the following sections.

1:3. Endotoxic Activities and Host Response to Lipopolysaccharide.

1:3:1. Physiochemical Aspects of Endotoxicity.

It has become clear that lipopolysaccharide (endotoxin) is the bacterial component responsible for the syndrome associated with septicaemia and septic shock. This has been demonstrated with animal models which have been challenged with purified LPS from any of a range of organisms, resulting in production of all of the pathophysiological changes observed in septicaemia. Recent evidence has also uncovered the presence of endotoxin at high levels in the circulation during episodes of septicaemia. Together, these two factors strongly indicate that LPS is the component of greatest significance in the processes involved in septicaemia.

The toxicity of LPS is expressed only upon its release from the bacterial cell surface, and not while it remains an integral component of the outer membrane of viable organisms. This accounts for the finding that bacteraemia itself does not necessarily lead to septic shock, but the presence of endotoxin in the blood (endotoxaemia) with or without bacteraemia does. The source of this endotoxin could either be bacteria which have been effectively treated with antibacterial agents resulting in cell lysis, or alternatively the large pool of endotoxin present in the intestine (see section 1:1:1).

The mode of presentation of lipopolysaccharide *in vivo* in an infected individual has an important bearing upon its potential toxicity as there are several forms in which LPS may be present in

the blood:

-Firstly, LPS can be found as an integral component of the bacterial cell, but, as mentioned above, cell-bound endotoxin cannot express toxic activities because exposure of the lipid A region is required for this purpose. It does appear that core-glycolipid epitopes may, however, be exposed to a great enough extent to exert immunogenicity im many individuals (Mackie et al 1982).

-Secondly, LPS may be found in fragments of outer membrane - or blebs - which have been sloughed off the cell during the process of normal growth in vivo as has been observed in some Klebsiella (Straus et al 1985; Straus 1987) and Pseudomonas cepacia (Straus et al 1988) infections as a response to metabolic conditions, although it has been observed that LPS is released from the cell surface as a by-product during proliferation of bacteria. Additionally, release of LPS in complexes has been observed for other organisms, including E. coli (Gankema et al 1980; Goris et al 1988; Hoekstra et al 1976; Mackowiak 1984; Morrison & Rudbach 1981; Rothfield & Pearlman-Kothencz 1969; Russell 1976; Tesh et al 1986; Tesh & Morrison 1988), Pseudomonas aeruginosa (Cadieux et al 1983), and S. typhimurium (Lindsay et al 1973; Mackowiak 1984; Rothfield & Pearlman-Kothencz 1969) when organisms are grown in vitro, and may therefore also occur during the infectious process. These complexes may thus also occur during growth in vivo, and further support for this exists as increases in endotoxin are observed during proliferation of bacteria in an experimental animal system (Shenep et al 1985a). It has also been determined that these complexes of OM may possess significant toxic activities, as observed for K. aerogenes (Straus 1987) and P. cepacia (Straus et al 1988), and may

thus contribute significantly to the development of septic shock.

-A third possibility is that LPS released from a cell upon lysis may form micelles through hydrophobic interactions between lipid A units. The relevance of micelles to the toxic activities is unsure, and it is more likely that LPS is released in complexes with other om structures.

-Serum lipoproteins appear to be of importance in the interactions of LPS with host modulatory systems. Of particular importance are high density lipoproteins (HDLP) which have been shown to bind rapidly to circulating free endotoxin (Abdelnoor et al 1982; Munford & Dietschy 1985; Munford et al 1982; Novitsky et al 1985; Tobias & Ulevitch 1983; Tobias et al 1985; Ulevitch & Johnston 1978; Ulevitch et al 1979; Ulevitch et al 1981; Warren et al 1987b), and many such complexes may be found. These LPS-HDLP complexes may, however, effectively neutralize toxicity (Warren et al 1986; Warren et al 1988), although a report of preservation of toxicity has been documented (Freudenberg et al 1980). HDLP-LPS complexes might only be found during the early stages of endotoxaemia when a host remains relatively healthy, with the levels of endotoxin rising once HDLP has been saturated. Two recent reports (Berger & Beger 1988; Konig et al 1988) have indicated that HDLP may play no significant role in the neutralisation of LPS toxicity. The role for HDLP thus remains doubtful, although other serum factors have been implicated (see above references).

Low density lipoprotein (LDLP) may also be of importance in the presentation of LPS *in vivo*, but reports of its relevance are few (Morel et al 1986; Navab et al 1988). It does appear, however, that LDLP allows LPS to retain full toxicity.

-Finally, LPS may be present as free molecules in the circulation, not complexed or bound in any way. It would be expected that LPS in this form would produce its greatest toxicity, but it would be difficult to demonstrate the presence of free LPS _in vivo_.

Another factor which has been shown to influence the toxicity of LPS is the nature of the cations which bind to the negatively charged groups present throughout the length of the molecule, and also the solubility of the molecule (Baggerman _et al_ 1987; Brade _et al_ 1987a; Csako _et al_ 1986; Galanos & Luderitz 1975; Galanos & Luderitz 1976; Goodman _et al_ 1984; Komuro _et al_ 1987; Ogawa & Kanoh 1984). The nature of the cations and solubility appear to be linked to the amphipathic nature of LPS, as alteration in toxicity by positively-charged ions appears to be the result of alterations in solubility of the LPS or LPS-complexes. For example, it has been determined that weakly positive ions, such as triethylamine, produce a highly soluble form of LPS which expresses high toxicity, probably as a result of exposure of lipid A. In contrast to this, when predominantly divalent cations are bound to the LPS molecule (Mg^{2+} or Ca^{2+}) the solubility of LPS is reduced, resulting in a lowered toxicity (which may be caused by LPS molecules forming complexes via ionic bridges and thus reducing exposure of lipid A). The ions which are present _in vivo_ would thus influence the relative toxicity of endotoxin.

LPS prepared from both smooth and rough strains of bacteria possess the toxic activities. The toxic moiety of LPS is the lipid A and the structural and conformational requirements for toxicity continue to

be uncovered (Galanos et al 1984b; Galanos et al 1985; Homma et al 1985; Matsuura et al 1985; Shimizu et al 1988; see also section 1:2). Lipid A itself is highly insoluble, and therefore its toxic activities are not fully expressed unless it is solubilised in some way. There is a requirement for at least the presence of a KDO unit (representative of Re LPS) to enable solubilisation and expression of toxicity. Solubilisation of lipid A can also be achieved by the use of various agents, including conjugation to bovine serum albumin (Galanos et al 1972; Ogawa et al 1986), incorporation into liposomes (Banerji et al 1979; Dijkstra et al 1987; Kataoka et al 1971), or alteration of ionic form (Galanos & Luderitz 1975; Galanos & Luderitz 1976), although all of these alter the toxicity in some way.

The toxicity of lipid A (and hence LPS) from different species has been shown to differ. LPS from Enterobacteriaceae has been shown to be far more toxic than LPS from Pseudomonas species, which is in turn more toxic than LPS from Bacteroides species (Johne et al 1987; Luderitz et al 1978). This effect seems to reflect differences in the structure of the lipid A from these organisms.

The size of the core oligosaccharide in LPS from rough mutant bacteria also affects the toxicity of LPS, most likely as a result of differences in the solubility of the molecule as a result of the presence of different sugar molecules. When only KDO units are substituted onto lipid A, lower toxic activity is obtained than when progressively more sugars are present (Morrison & Rudbach 1981). The size of O-antigen has also been shown to affect solubility and

toxicity of free lipopolysaccaride (Cryz *et al* 1984b). The number of sugars substituted onto lipid A and the size of the O-antigen thus affect the solubility of LPS and, as a result of this, its toxicity.

The expression of toxicity of LPS is a complex process under the influence of many factors, but despite this, LPS has been shown to possess a wide range of activities both *in vivo* and *in vitro*.

1:3:2. Toxic Activities of Endotoxin and Pathophysiology of Septic Shock.

LPS binds non-specifically to most cells and tissues - probably through hydrophobic interactions and by binding to receptor sites - as well as to humoral factors (Bradley 1979; Braude 1980; Cybulsky *et al* 1988; Dinarello 1983; Freudenberg *et al* 1982; Kalter *et al* 1985; Luderitz *et al* 1984; Mathison & Ulevitch 1979; Morrison 1983; Morrison 1987; Morrison & Rudbach 1981; Morrison & Ryan 1979; Morrison & Ulevitch 1978; McCartney & Wardlaw 1985; Nowotny 1987; Rietschel & Brade 1987; Rietschel *et al* 1984b; Rubenstein *et al* 1962; Wolff 1973; Zimmerman & Dietrich 1987). Because it interacts with so many host systems, LPS is a potent effector molecule, resulting in the release and/or activation of many endogenous mediators. The host factors involved include complement (via classical and alternate pathways) (Goldstein 1985; McPhaden & Whaley 1985), prostaglandins, interferons, platelet activating factor (Hsueh et *al* 1987; Wallace 1987), interleukins (Keppler *et al* 1987; Northoff *et al* 1987; Urbaschek & Urbaschek 1987), Hageman factor, and cachectin (tumour necrosis factor) (Bauss *et al* 1987; Beutler & Cerami 1987; Cerami & Beutler 1988; Mannel *et al* 1987; Michie *et al*

1988; Old 1987). LPS can also directly affect the action and function of phagocytic cells, leukocytes and platelets (Baker _et al_ 1988; Groenveld _et al_ 1988; Haeffner-Cavillon _et al_ 1985; Luscher 1987; McCuskey _et al_ 1987; Parker & Parillo 1983; Regel _et al_ 1987; West _et al_ 1985).

As a result of massive activation and alteration of the humoral and cellular immune systems, the complement system, as well as the other systems involved, a febrile condition is induced, which leads to reduced blood pressure, leukocytosis and leukopenia, thrombocytopenia, alteration of metabolism, and release of further mediators. This condition may then proceed further to produce hypovolaemic shock, respiratory oedema, respiratory collapse, and disseminated intravascular coagulation (Ali _et al_ 1987; Al-Sarraf _et al_ 1988; Cybulsky _et al_ 1988; Fowler _et al_ 1983; Gathiram _et al_ 1987a; Gelin 1980; Harris _et al_ 1987; Luce 1987; McCartney & Wardlaw 1985; Rogers-Jacob & Bone 1986). These later stages of septicaemia result in a syndrome which is called septic shock and which, in the majority of cases, results in death.

The activation of the complement system by endotoxin has been studied in great detail and appears to be of central significance in the initiation of the febrile state which accompanies septicaemia (Goldstein 1985; McPhaden & Whaley 1985; Vukajlovich 1986; Vukajlovich _et al_ 1987). Upon release of endotoxin from bacteria, complement is activated by both the classical and alternative pathways, causing release of febrile mediators and immune activators.

The means by which LPS activates the other cellular and humoral mediators of toxicity is unclear, but it is believed that some toxic effects may result from disruption of cellular membranes by lipid-A (Kilpatrick-Smith et al 1985; Shands 1973), or by causing release of cellular contents. There is also evidence that LPS localises intracellularly in the nucleolus of cells and may thus alter protein synthesis (Lucas et al 1985) causing cell lysis or possibly release of immune system mediators or possibly even through the formation of immune complexes contributing to tissue damage (Ohshio et al 1988). LPS may also affect intracellular enzymes, resulting in the alteration of normal cellular metabolism and production of immune mediators. The normal immune response may thus be amplified by the high levels of LPS present in the circulation.

Lower doses of endotoxin do not produce the above detrimental effects, but do, on the contrary, result in beneficial effects (Morrison 1983; Ribi 1984; Urbaschek & Urbaschek 1987). LPS is a non-specific B-cell mitogen and can produce a general increase of antibody levels to many antigens (Ribi et al 1987), and in relation to this, LPS is a potent adjuvant, causing amplification of immune response to various antigens. A clearer example can be observed after either Gram-negative infection or challenge with non-lethal doses of endotoxin or endotoxin precursors. These can produce a non-specific resistance to subsequent infection (Chase et al 1983; Chong & Huston 1987; Vuopio-Varkila et al 1988b) and could be used to modify the immune response of individuals at high risk of infection. Low doses of LPS have also been shown to possess

anti-tumour activity through induction of cachectin (tumour necrosis factor - TNF) (Carswell et al 1975; Freudenberg et al 1984; Ribi et al 1982; Ribi et al 1984). LPS therefore possesses potentially therapeutic activities and much work is being carried out at present in assessing its role and the role of structural analogues as a therapeutics agent in septicaemia and certain other clinical situations.

The role of lipopolysaccharide as a mediator of toxicity in diseases does not stop with septicaemia. Its role in several diseases has been confirmed, including Toxic Shock Syndrome (de Azavedo et al 1985), Adult Respiratory Distress Syndrome (Fain et al 1983; Fowler et al 1983; Kaplan et al 1979), renal failure (Bailey 1976; Wardle 1982; Wardle & Wright 1981), liver disease (Young et al 1986), heat stress (Gaffin et al 1981), radiation sickness (Gaffin et al 1987), and a putative role has also been suggested in production of tissue damage in periodontal disease (Lucas et al 1985). It may well turn out that LPS is a far more important mediator of disease in many more situations than is known at present.

Irrespective of its mechanism of activation and modification of host immune response, LPS is undoubtedly a highly active toxin of great importance in septicaemia, and as a result of its diverse actions it is central to the development of septic shock.

1:3:3. Host Response to Endotoxin.

Not every person is susceptible to systemic infection by Gram-negative opportunistic pathogens (see section 1:1:2 for

pre-conditions) and most people are capable of mounting an immune response to the causative organisms and/or endotoxins. The effectiveness of this response has an important influence upon the outcome of septicaemia.

All factors of the immune system have been found to be important determinants of the outcome, and ineffectiveness of any of these could result in an overwhelming bacteraemia leading to septicaemia and septic shock (as discussed in section 1:1:2). Many of the symptoms of septicaemia and septic shock do, however, result from massive activation of the immune system by LPS as mentioned previously. The balance between appropriate protective response and overactivation of the immune system is thus finely divided.

In a "normal" individual, invasion of the blood is usually prevented by the non-specific host defences present on the mucous membranes and skin. When organisms do penetrate these surfaces, there are many factors which can prevent overwhelming infection of the blood. These include removal of bacteria from the circulation by the reticuloendothelial system (RES) and other phagocytic cells, destruction of bacteria inside phagocytes, opsonisation of bacteria and neutralisation of bacterial products by antibodies, and activation of the complement cascade by either classical or alternative pathways, resulting in bacteriolysis.

It appears that the presence of bacteria up to certain levels can be tolerated, but if numbers exceed a threshold value, septicaemia may result. The development of septicaemia most probably occurs as a

result of a blockade of the RES by the sheer number of bacteria present, massive activation of complement, and removal of protective antibodies. This would prevent further removal of bacteria or their products and permit an increase in levels of bacteria and endotoxin in the blood, leading to expression of endotoxicity.

Alternatively, if bacteria are efficiently lysed by the host immune mechanisms (complement and antibody) or by effective treatment with antimicrobial agents, endotoxin is released and could then proceed to activate mediators of toxicity (as described above) if not removed from the circulation.

One further possibility resulting in release of endotoxin would be through application of therapeutic agents which (either directly or as a side-effect) alter the permeability of the intestinal lumen thereby allowing entry of endotoxin into the circulation. Once again this would result in expression of toxic activities of LPS if not rapidly removed. This could additionally be achieved during ischaemic damage to the bowel or tissue perfusion, thereby releasing vast amounts of endotoxin (Freeman & Gould 1985a).

It can be seen that in a non-compromised individual large numbers of bacteria or large amounts of endotoxin would thus be required to induce septicaemia, as removal of all but very high concentrations of bacteria and LPS could be obtained. As a result of an immunocompromised state (whether by depletion of complement, antibody, phagocytosis, or cell-mediated immunity), this condition could be achieved more rapidly. Compromised patients are therefore

observed to enter into septicaemia at a higher rate.

Even in a healthy person, endotoxin is constantly seeping from the intestine into the blood, but is removed by phagocytic cells in the liver before it can mediate its toxicity. Despite the removal of endotoxin, an antibody response can be generated.

Much evidence points to the role of the liver (and to a lesser extent, the spleen) in clearance of bacteria and endotoxin and their release into the circulation. It has been shown that a high rate of clearance by phagocytic cells in the liver (hepatocytes) correlates well with survival from septicaemia, while a lowered efficacy of clearance is linked with a poorer rate of survival (Billiar *et al* 1988; Cheslyncurtis *et al* 1988; Freudenberg *et al* 1982; Jacob *et al* 1977; Katz *et al* 1984; Lumsden *et al* 1988; McCuskey *et al* 1987; Prytz *et al* 1976; Shirai *et al* 1988; Thompson *et al* 1988). Any means by which this important function of the liver is depleted would thus allow entry of LPS into the circulation from the intestine. Therefore liver dysfunction resulting from underlying disease, therapy, or the action of certain toxins may reduce the liver's ability to remove LPS and bacteria and detoxify endotoxin, thereby permitting development of septicaemia and septic shock.

Other phagocytic cells and organ systems also have important roles to play in the removal of bacteria and/or endotoxin. Phagocytic cells provide a non-specific defence system which can provide protection against systemic bacterial or endotoxin challenge (Hammer *et al* 1981; Regel *et al* 1987; Vuopio-Varkila 1988; Vuopio-Varkila &

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Makela 1988). Polymorphonuclear leukocytes (PMNL) and macrophages have both been demonstrated to sequester endotoxin, and have been shown to possess activities which alter the LPS molecule, and which may also reduce its toxicity (Duncan & Morrison 1984; Farley et al 1988; Freudenberg & Galanos 1986; Freudenberg & Galanos 1988; Freudenberg et al 1985; Munford & Hall 1985; Munford & Hall 1986; Weinstein & Young 1976; Young & Armstrong 1972). In addition, PMNL have been shown to possess a protein which causes lysis of Gram-negative bacteria (Hovde & Gray 1986a; Hovde & Gray 1986b; Tedesco et al 1986; Veld et al 1988). Thus phagocytic cells provide further means of protection from systemic infection, and have an important role in Gram-negative septicaemia.

Exposure of Gram-negative bacteria to normal human serum in the absence of specific antibodies, results in the release of lipopolysaccharide from cells and reduction in the number of viable organisms (Tesh & Morrison 1988; Tesh et al 1986). This appears to occur through the action of complement (C') upon cells, and this system is central in the prevention of systemic bacterial invasion. Complement possesses both lytic and opsonic activities for intact Gram-negative bacteria via both the classical and alternative pathways (Ihara et al 1982; Loos & Clas 1987; Loos et al 1978; McPhaden & Whaley 1985; Rowley 1973; Rozenberg-Arska et al 1986; Schiller 1988; Sculier & Klastersky 1984; Taylor 1983; Wright & Levine 1981; Vukajlovich 1986). The C' system is effective against many strains of organisms, though there are some which can prevent effective action of complement (see section 1:2). Complement therefore has an important role both by itself and in conjunction

with other components of the immune system, for the lysis and removal of organisms from the circulation.

Within the population a wide range of antibodies of IgG, IgM, and IgA classes have been detected which recognise Gram-negative bacteria and their lipopolysaccharides. A wide range of levels of these antibodies to a variety of O-antigen, core oligosaccharide, and lipid A components of lipopolysaccharides have been shown to exist (G.R. Barclay - unpublished data; Appelmelk et al 1985; Appelmelk et al 1986b; Appelmelk et al 1987a; Barclay & Scott 1987; Brade & Galanos 1983; Braude 1980; Brauner et al 1987; Cohen & Norins 1966; Fomsgaard et al 1987a; Gleeson et al 1987; Jacobson et al 1987; Kataoka et al 1971; Law & Marks 1985; Marget 1987; Mattsby-Baltzer & Alving 1984b; Mattsby-Baltzer & Kaijser 1979; Nys et al 1987; Nys et al 1988; Schedel et al 1987; Scott & Barclay 1987; Stoll et al 1985; Stoll et al 1987; Vanesian et al 1987; Young 1972; Young et al 1975b). Antibody titres have been shown to rise from birth - as a result of production of antibodies against organisms which form the commensal flora (Chedid et al 1968) - up to the age of three. After this age, titres remain fairly stable (Law and Marks 1985). It has also been observed that, within individuals in a blood donor population, levels of antibodies to a particular lipopolysaccharide remain fairly stable over a period of up to twelve months (G.R. Barclay unpublished results).

Because O-antigen is the outermost component of LPS, and also the major antigenic structure, antibodies to many O-antigens from organisms which cause septicaemia (and others that do not) can often

be found at high levels in healthy people. Despite the prevalence of antibodies to O-antigen, antibodies to core glycolipid (CGL - comprising lipid A and core oligosaccharide) have also been detected in all individuals studied.

Anti-O-antigen antibodies are specific to a particular O-antigen, although some limited cross-reactivity can be observed within and between some organisms (section 1:2). Anti-core glycolipid (anti-CGL) antibodies show far greater cross-reactivity between different species and genera, but these antibodies are present at lower levels than the O-antigen-specific antibodies. In spite of the lower concentrations of anti-CGL antibodies, they may perform an equally important role in protection against the effects of systemic release of Gram-negative bacteria or endotoxin.

Core glycolipid is the LPS component against which naturally cross-reactive antibodies can be detected. Antibodies to outer core possess the ability to cross-react with other Gram-negative bacteria because of the similarities observed in outer core structures (see section 1:2) although this ability is restricted because of structural heterogeneity. Inner core structure is more highly conserved between genera, and consequently antibodies directed against inner core are highly cross-reactive. Additionally, antibodies to lipid A can also be detected at low levels, and these also would be expected to possess cross-reactivity.

Antibodies to lipopolysaccharides have been shown to possess antibacterial properties of relevance in septicaemia by three main

means: firstly, antibodies can perform opsonic activities, either directly or via complement, enabling uptake of cells or endotoxin into phagocytes; secondly, antibodies can result in bacteriolysis by activation of complement, thereby releasing endotoxin; and finally, antibodies may act in an anti-endotoxic capacity, neutralising or modulating the activities of LPS. The first two functions are performed mainly by antibodies directed against the O-antigen of LPS (Dunn 1988; Frank et al 1987; Gaffin & Wells 1987; Just et al 1987; Kim et al 1988; Lam et al 1987a; Lam et al 1987b; McIntyre et al 1986b; Pudifin et al 1985; Sawada et al 1987; Young 1984), and appears to be a lesser role for anti-core antibodies. In contrast, reports indicate that antibodies to core glycolipid can bind to free lipid A and CGL, but variation is seen in the ability to bind to whole organisms (Appelmelk et al 1988; Elkins & Metcalf 1985; Gigliotti & Shenep 1985; Miner et al 1986; Teng et al 1986). Anti-CGL possess only limited opsonic and bactericidal ability, if any at all (Betz et al 1982; Crowley et al 1982; van Dijk et al 1981; Kirkland & Zeigler 1984; Mehta et al 1988; Michael & Mallah 1981; Miner et al 1986; Rietschel & Galanos 1977; Vreede et al 1986; Welch et al 1979; Young 1984; Young & Stevens 1977; Young et al 1975a), and perform mainly antitoxic activities through binding to epitopes within lipid A and/or core, and inhibiting binding of endotoxin to humoral and cellular targets, thereby preventing activation of the immune system. Anti-O-antigen antibodies may also perform anti-toxic activity by enabling removal of LPS in some of its possible forms (see section 1:3:1) from the circulation.

Antibodies directed against lipopolysaccharide therefore have a

central position in the prevention of development of septicaemia, and depletion of anti-LPS antibodies could permit progress of this condition.

Further support for the central role of antibodies in septicaemia has been obtained in a series of clinical studies. Antibodies to O-antigen and core glycolipid endotoxin have been shown to fall during the acute phase of septicaemia, but rise in patients that survive (Nys et al 1988; Peter et al 1979; Pudifin et al 1985; Schedel 1988). Decreasing levels of antibodies are associated with patients who ultimately die, while patients maintaining elevated levels of antibodies, or with increasing antibody levels show a far higher rate of survival (Brauner et al 1986; Brauner et al 1987; Cohen et al 1987; Freeman & Gould 1985b; Freeman & Gould 1986; Matthews et al 1986; McCabe et al 1973; Nys et al 1987; Pollack et al 1983a&b; Young 1972). In relation to this, patients who possess high levels of antibodies prior to septicaemia have been shown to show a lower infection rate and a higher survival rate than those with negligible levels of antibody (Pollack & Young 1979; Stoll et al 1985).

The above observations therefore re-inforce the highly important role of anti-LPS antibodies in septicaemia and endotoxaemia, and elevation of antibodies to LPS may be of great significance in the prevention and reduction of symptoms of septicaemia, although antibodies may in some cases lead to production of circulating immune complexes which may lead to tissue damage (Ohshio et al 1988).

Other serum factors also have a role to play in the prevention of septicaemia. Several factors have so far been discovered in various species of vertebrates, including humans. These include factors which bind endotoxin resulting in neutralization of activity, and also proteins which effect bacteriolysis. It should be noted that these factors cannot be ignored as they appear to be important in resistance and immunity to endotoxin. Among these components are high-density lipoprotein (Munford et al 1982; Novitsky et al 1985; Ulevitch & Johnston 1978; Warren & Chedid 1987), although the role of this has recently been queried (Berger & Beger 1988; Konig et al 1988). Low molecular weight proteins (Brade and Brade 1985; Tobias & Ulevitch 1983; Tobias et al 1985; Tobias et al 1986) which bind and alter toxicity of LPS have also been implicated, as have a bactericidal protein (Farley et al 1988), and a macroglobulin (Michael & Rosen 1963). In addition, several other factors have been demonstrated to possess LPS-binding properties, and may also play important roles in neutralisation and/or removal of endotoxin from the circulation (Bailey 1976; Berger & Beger 1988; Cahill 1983; Jirillo et al 1986; Johnson et al 1977; Warren et al 1985; Yamaguchi et al 1986; Yamaguchi et al 1987). These factors appear to possess endotoxic modulating activity, but their relevance to the human situation is not clearly defined at present, as most have so far been discovered in other species.

Many factors are obviously involved in protection of individuals from the deleterious effects of septicaemia or endotoxaemia, but it is obvious that despite these factors, many cases are still

observed. The difference between appropriate response and over-reactivity, resulting in septic shock, is finely balanced and dependent upon both host and pathogen factors. As the host components responsible for the prevention of sepsis become clearer, and in view of the severity of this syndrome, it is necessary that appropriate preventative and therapeutic measures are available to reduce or remove the threat of fatalities caused by Gram-negative septicaemia. Those measures which are currently available, as well as emerging regimens, are discussed in section 1:5.

1:4. Small Animal Models of Gram-negative Septicaemia and Endotoxaemia.

Because of the severity of the syndrome associated with septicaemia, it has become necessary to develop animal models to evaluate the effects of systemic infection by Gram-negative bacteria and their endotoxins, and to assess potential therapeutic regimens. A wide range of animal models has been developed, each possessing different physiological and immunological responses to bacterial or endotoxin challenge.

The species of animal which is most reactive to endotoxin is man (Greisman & Hornick 1969; Michie et al 1988; Westphal 1975; Wolff 1973). It has been shown that man is sensitive to nanogram (10^{-9} gram) or even picogram (10^{-12} gram) amounts of LPS per millilitre of blood. Reactivity to these levels of lipopolysaccharide is observed even in healthy individuals. Animal models can therefore only be used to give an indication of effects of bacteria or LPS or protective activity of agents in humans.

Many animal species have been used to determine various factors involved in septicaemia, but in most cases very high doses of bacteria and/or endotoxin, in comparison to those needed in humans, have been required to produce the symptoms of septicaemia or death resulting from septic shock. The animal of closest sensitivity to that of humans is the rabbit and it has found use in several models.

As a result of the lower sensitivity of other animal species to

endotoxin and bacteraemia, it has been necessary to induce a state of compromise in these animals. Compromise of animals by a variety of means has led to a reduction in challenge doses required to produce septic shock. In addition, in some instances the mode of compromise has produced a condition in the animals similar to conditions which permit septicaemia in hospital patients. Induction of a state of compromise in animals can therefore result in production of models of greater relevance to the evaluation of septicaemia and therapeutic strategies.

The animals which have found most usage as models of septicaemia or endotoxaemia include mice, rats and rabbits, and to a lesser extent, cats, dogs, sheep, pigs, and primates. Two general models have come into use:

i) endotoxaemia, in which animals are challenged with endotoxin purified from bacterial cells,

and ii) infection, in which live bacteria are used to challenge the model. Additionally, several modes of compromise of animals are also in use as described below.

1:4:1. Models of Endotoxaemia.

Endotoxin can be found at high levels in the blood and tissues of patients with septicaemia. Thus it seems appropriate that challenge of model species with lipopolysaccharide would be a relevant means of mimicking the systemic effects of endotoxin. It has indeed been shown that challenge of animals with endotoxin results in production of the symptoms associated with septic shock, therefore challenge with endotoxin does appear to be relevant for the assessment of

septic shock.

Mice and rats have received greatest attention as models for endotoxaemia. A wide range of sensitivities to LPS has been obtained in different strains of these animals, with doses of up to 2mg being required to produce symptoms in some strains.

Actinomycin D Model.

Treatment of mice or rats with actinomycin D prior to challenge has produced an increase in sensitivity to the toxic effects of lipopolysaccharide of greater than 100000 fold (Brown & Morrison 1982; Dunn et al 1986; Pieroni et al 1970). After treatment with actinomycin D it has bee shown that it is possible to reduce the dose required to produce 50% lethality (LD50) from approximately 200ug to 10^{-3}ug. Other strains of animals show reduction of doses required for LD50 to 30ug or 150ug, although the factor of reduction remains approximately 10^5. Actinomycin D acts by potentiating the reactivity of host systems to LPS activation by a mechanism which is not clear. This has allowed reduction of the challenge dose required to produce pathophysiological changes in the animal models.

Galactosamine Model.

One other means of compromise which has found usage in mice or rabbits is the reduction of efficient removal of LPS in the liver through treatment with **D**-galactosamine (Freudenberg et al 1986; Freudenberg & Galanos 1988; Galanos et al 1979; Lehmann et al 1987). This aminosugar affects hepatocyte function and depletes their capacity to efficiently perform their phagocytic function. This

therefore permits levels of endotoxin in the circulation to remain high, resulting in prolonged expression of LPS toxicity, and increased pathophysiological alterations in the animals. As a result of the persistence of high levels of LPS, the LD50 is reduced from 200-300ug to 1-5ng (a factor of 10^5) dependent upon the strain of animal.

Schwartzman Reactions.

The local Schwartzman reaction in rabbits has been used as a means of determining toxicity of LPS for many years (Ito et al 1985; Ziegler et al 1973b). In this model, non-compromised rabbits are challenged intradermally with non-lethal doses of lipopolysaccharide. After 24 hours, animals are then challenged intravenously with a second non-lethal dose of LPS which produces dermal necrosis at the original sites of challenge - a reaction which is referred to as the local Schwartzman reaction. This reaction is representative of the disseminated intravascular coagulation (DIC) reaction observed during the latter stages of septicaemia (see section 1:3:2). In this model intravascular coagulation occurs locally at the site of initial challenge and produces necrosis of the tissues. This model can also be used to assess the protective capacities of anti-LPS antibodies.

The general Schwartzman reaction is a similar model to the above. In this case the initial challenge is given intravenously, which produces systemic DIC upon challenge with the second dose of LPS.

Other Models of Endotoxaemia.

Additionally, models using sheep, pigs, cats, or dogs, or even chick embryoes have also been used, but to a lesser extent. There are also several other means of sensitisation of animals to the actions of endotoxin, as discussed by Galanos et al (1986), Pieroni et al (1970) and Wichterman et al (1980), but those mentioned above represent the most widely used.

Each of the models of endotoxaemia has been shown to mimic only certain aspects of septic shock in humans, but nevertheless they do provide useful insights into the many activities of endotoxin and anti-endotoxic molecules.

1:4:2. Models of Infection.

Models of infection have found wider application than models of endotoxaemia. Once again rodents have been used most commonly and a variety of modes of immunocompromise has been applied.

Some strains of rodents have been used without any means of compromise. This does, however, require very high challenge doses of bacteria (often over 10^7 organisms) in order to produce a febrile state and septic shock. This causes difficulty in assessment of physiological responses and potential therapeutic agents because of the very high bacterial concentrations in relation to those found in cases of septicaemia.

Neonatal rats have found use in assessment of virulence of Gram-negative bacteria in systemic infections and in determination

of effectiveness of antibody therapy (Hill & Bathras 1986; Kim & Anthony 1983; Raff et al 1988; Saukkonen, Nowicki & Leinonen 1988). This again has required very high doses of bacteria to mimic the effects observed in humans. A similar rat model has also found use in assessment of physiological changes during septicaemia.

As a result of the high resistance to the effects of endotoxin or Gram-negative bacterial challenge, it has become necessary to induce a state of compromise in animals used as models of septicaemia as in models of endotoxaemia. Several means of compromise of these animals have been developed, either to reduce the immune competence of animals or to increase the virulence of challenge organisms.

Models of Neutropenia.

A state of sub-lethal neutropenia induced in an animal results in a greatly increased susceptibility to bacterial challenge (Raff et al 1988). Treatment of mice with cyclophosphamide results in profound neutropenia within 5 days. This results in a marked lowering of challenge dose required to produce fatal septicaemia (from 10^4-10^5 organisms to below 100) and thus easier assessment of therapeutic agents.

Prior development of a similar neutropenic model by Collins et al (1986) where bacteria are inoculated into a local wound, followed by the application of antimicrobial agents in addition to immunoglobulins has also produced an effective means of assessing the therapeutic activity of antibodies. This model results in a local infection which proceeds into septicaemia over a period of

time, and may thus be a good representations of septicaemia as observed in some clinical situations.

Another neutropenic model which may mimic the natural course of septicaemia is that of Ziegler et al (1973a) in which rabbits are rendered neutropenic by treatment with nitrogen mustard. This permits invasion of the circulatory system by bacteria which were previously administered to the animals and were permitted to colonise the intestine. Induction of neutropenia permits invasion of bacteria from the intestine followed by their proliferation, resulting in a febrile condition which proceeds in many cases to death.

A similar model to the above has been described (Teng et al 1988) in which neutropenic rabbits are inoculated intra-ocularly with challenge organism (in this model P. aeruginosa), producing a serious local infection which proceeds to septicaemia.

A further model in which neutropenia is induced has been developed recently by Zweerink and others (1988). In this model mice with a congenital immunodeficiency state (resulting in an inability to produce antibodies to carbohydrates and other antigens), were rendered neutropenic. These mice were then challenged with Pseudomonas aeruginosa and treated with monoclonal antibodies. This produced a model which reduced challenge doses of bacteria required to produce septic shock, and seems to represent another model which parallels certain clinical conditions which may lead to septicaemia.

Models with Mucin and/or Haemoglobin.

Increase of the virulence of bacteria by the use of mucin and haemoglobin (Appelmelk et al 1986; Marks et al 1982) or mucin alone (Coughlin & Bogard 1987) has permitted the reduction of lethal dose to very few organisms (less than 100 organisms in most cases) and a concomitant improvement in assessment of protective activity of anti-LPS monoclonal antibodies in these models. Haemoglobin permits growth of bacteria, probably by acting as a source of iron for the organisms (Dunn et al 1983a; Dunn et al 1983b; Dunn et al 1984).

Other Models of Infection.

Wichterman et al (1980) have described a model of septicaemia with normal intestinal flora. This is produced by puncturing the caecum of neonatal rats which develops into peritonitis and septicaemia. Many parameters of infection can be studied including potential therapeutic strategies. This model is said to possess many of the features of septicaemia in man and is thus of particular relevance to the study of septicaemia. Further means of bacterial challenge are also discussed by Wichterman.

There are many animal models of septicaemia and endotoxaemia currently in use, a small proportion of those commonly used being discussed above. No model can be said to adequately mimic the situation observed in septicaemic patients, but the models do provide valuable insights into the processes involved in in the actions of endotoxin and bacteria upon living organisms. A highly

valuable use of animal models (and the reason for which many of the above mentioned models were developed) is for the assessment of potential therapeutic agents, and many of the therapeutic agents mentioned in section 1:5 have been assessed by using some of these models.

1:5. Therapy and Prophylaxis for Septicaemia caused by Gram-negative Bacteria.

As a result of the high fatality rate of Gram-negative septicaemia it has been necessary to develop preventive and therapeutic strategies to cope with this problem. Many such measures have been taken, with a resultant variation in prevention of fatalities. Means which have found use are antimicrobial agents, immune system modulators, and preparations of antibodies, as well as means of physiological resuscitation. Many of the problems encountered in septicaemia occur as a result of inappropriate or inadequate therapeutic or preventive measures. It is thus essential that the most relevant strategies are employed to prevent loss of life.

1:5:1. Antimicrobial Therapy of Gram-negative Septicaemia.

There are many antimicrobial agents with proven efficacy against Gram-negative bacteria, and which have therefore found application in the treatment of septicaemia and other infections (Allen & Moellering 1985; DeMaria et al 1985; Esquembre et al 1987; Kreger et al 1980b; Pizzo & Young 1984; Stutman et al 1986; Verhagen et al 1986; Young 1985a; Young 1985b). Wide variation in the success of these agents has been described by many authors, with only limited protection observed in many reports. Therefore the usefullness of most antimicrobial agents is restricted. In fact, many of the fatality rates noted in table 1:2, section 1:1:1, were obtained during administration of antimicrobial agents.

There are some reports of higher efficacy with certain agents, but

again the "cure" rate remain fairly low with occassional reports of high efficacy (Baruchel _et al_ 1986; Daenen & de Vries-Hospers 1988; Gathiram _et al_ 1987b; de Jongh _et al_ 1985; McKellar 1985; Pierard 1986; Rolin & Bouanchaud 1986; Warren _et al_ 1985a; Weinstein 1986). Even lipopolysaccharide-binding antimicrobial agents - cationic polypeptides such as polymyxin - are of limited use because of their toxicity to animals (Peterson _et al_ 1985b; Peterson _et al_ 1987; Rocque _et al_ 1988; Warren _et al_ 1985a), although there have been instances where polymyxin has been shown to reduce certain metabolic alterations seen in experimental septic shock (Flynn _et al_ 1987).

Newer agents which directly affect synthesis of lipopolysaccharide have been shown to possess strongly anti-Gram-negative activity (Goldman _et al_ 1987; Hammond _et al_ 1987). Many aspects of these compounds have yet to be assessed, but these antimicrobial agents are potentially of use in the therapy of septicaemia.

Although an antimicrobial agent may be effective both _in vitro_ and _in vivo_, this does not ensure survival of patients. A probable reason for this is that it is the endotoxin of the cell wall which is causing the alterations in patients and not intact bacteria themselves, and antimicrobial agents are not directed towards LPS. Evidence has recently been produced by several groups of researchers which has shown that the antibacterial agents may actually be directly involved in the pathogenesis of septic shock. Shenep and co-workers (Shenep & Mogan 1984; Shenep _et al_ 1985b) have determined in a rabbit model of infection that bactericidal antibiotics are effective in lowering the numbers of organisms present in the blood

by lysis of the bacteria, but this causes a resultant increase in the level of endotoxin. Bacteriostatic antibiotics, on the other hand, have little effect on bacterial concentration whilst endotoxin levels remain stable. This means that, when applied to humans, either approach could therefore lead to worsening of the patients condition rather than improvement. Further groups have also shown that LPS is released in large quantities by antimicrobial agents, both in vivo and in vitro (Cohen & McConnel 1985; van Deventner et al 1988; Editorial 1985; Freeman 1980; Goto & Nakamura 1980; Hopkins 1977; Hopkins 1978; McConnell & Cohen 1986; Tauber et al 1987). Antibacterial agents must thus be chosen carefully to minimise the possibility of detrimental effects caused by release of endotoxin.

A further adverse role for antimicrobial agents in the pathogenesis of septicaemia exists. A large proportion of bacteria isolated in cases of septicaemia (whether from endogenous or environmental sources) have been determined to have acquired increased resistance to the most frequently applied anti-bacterial agents (Acar 1985; Finland 1977; Lacey 1984). This means that infection by bacteria with pre-existing resistance occurs, therefore reducing the range of agents which can be used.

The actions of antimicrobial agents are not all deleterious, and many have been shown to aid the immune system in removal and/or lysis of bacteria. Many studies have shown that the expression of LPS on the bacterial surface is altered when organisms are grown in the presence of sub-lethal doses of antimicrobial agents, which permits easier access of antibodies and therefore enhanced

complement activation. This results in increased bacteriolysis, phagocytosis and intracellular killing (Dalhoff et al 1986; Overbeek et al 1987; Trautman et al 1985; Wiemer et al 1985; Williams 1987). It has also been shown that a combination of antimicrobial agents and anti-LPS antibodies act synergistically in experimental models of septicaemia (Collins et al 1986; Greisman et al 1979; Neeley & Holder 1987).

Antimicrobial agents therefore possess actions which are both beneficial and deleterious, and use of these agents must be carefully assessed. Despite the potential for releasing endotoxin, antimicrobial agents may, however, provide a useful adjunct to immunological therapeutic strategies.

1:5:2. Other Therapeutic and Preventive Strategies.

In addition to application of antimicrobial agents, there are non-anti-bacterial strategies which are employed under various situations. When septic shock occurs it is necessary for many patients to receive supportive care throughout the acute stages. The management of many septic shock patients requires fluid and oxygen resuscitation, application of immunomodulatory drugs and vasoactive agents, and replacement of reticulo-endothelial factors, as well as other physiological modifiers in addition to antimicrobial agents (Karakusis 1986; Ledingham & McArdle 1978; Ledingham et al 1984; Ledingham et al 1988b; Luce 1987; Wolff 1982; Young 1985c; Zimmerman & Dietrich 1987). These modes of support have produced at best only minimal benefits. They may, however, be useful adjuncts to other therapies as they provide support to a patient with lowered immune

competence, but do not aid in the removal or neutralisation of the mediators of septicaemia. As these measures rely upon an already weakened host immune system to effect recovery, they cannot be regarded as a reliable therapeutic regimen, although as knowledge of the processes of septic shock advances, the requirements for effective resuscitation are becoming clearer.

The success of resuscitative and supportive measures in preventing fatalities has been shown to be dependent upon the timing of their initiation. If applied early after onset of a febrile state, a higher rate of success is observed than if initiation of supportive care is delayed (G. Ramsay - unpublished results). This phenomenon may have an important influence on the outcome for any mode of therapy.

One means which has been shown to reduce the frequency of septicaemia is selective bowel decontamination (SBD) in which a range of oral antibacterial agents is given to patients at known risk of septicaemia prior to surgery (Alcock & Ledingham 1988; Karp et al 1988; Ledingham et al 1988b; Schmeiser et al 1988; van der Waaij 1988; Wells et al 1987; Wiesner et al 1988). This results in removal of most potentially pathogenic aerobic and facultatively anaerobic commensal organisms, which results in a fall in the number of cases of septicaemia from this endogenous source. This, however, does little to remove the threat posed by septicaemia from external sources or from organisms which are able to recolonise the intestine (which may be resistant to antimicrobial agents - see above). Despite these potential drawbacks, SBD is a useful method for the

reduction of the incidence of infection.

Removal of endotoxin from blood followed by re-infusion of the blood has been attempted in an animal model (Cohen et al 1987b) and in endotoxaemic neonates (Togari et al 1983). The animal model involved plasmapheresis during which endotoxin was removed by adsorption onto immobilised polymyxin, resulting in removal of endotoxin and removal of fatal outcome. The neonatal patients were given exchange blood transfusion, and complete recovery of patients was achieved. These processes may therefore provide another measure which may have some application in septicaemia or endotoxaemia, but their effectiveness in relation to number of patients treatable must be doubtful.

Neutralisation of mediators of endotoxin activity have been shown to be effective in the treatment of endotoxin shock. Administration of monoclonal antibodies directed against tumour necrosis factor (TNF) in animal models resulted in lessening of pathophysiological alterations and reduction in fatalities (Beutler et al 1985; Mathison et al 1988; Shimamoto et al 1988; Tracey et al 1987). Despite their protective capabilities, these agents fail to remove the initial stimulus of septic shock - endotoxin - but again may prove to be a useful adjunct to other therapeutic strategies.

Components of serum other than antibodies are also involved in endotoxin removal and/or neutralisation (see section 1:3). These factors cannot be ignored as potential therapeutic agents as they may form part of a future strategy against endotoxaemia. In addition, precursors and synthetic analogues of lipid A have been

shown to possess anti-endotoxic activities (Danner _et al_ 1987; Golenbock _et al_ 1987; Golenbock _et al_ 1988; Proctor _et al_ 1986) and may also prove to be therapeutically useful once their activities are fully assessed.

None of the above strategies have proven to be highly effective in preventing fatalities from septic shock caused by Gram-negative bacteria. This occurs as a result of failure to remove or neutralise the initial stimulant (endotoxin) of the pathophysiological changes observed in this condition. A great deal of interest has therefore been directed towards agents which remove and/or neutralise endotoxin. Anti-LPS antibodies have been given most attention as they are central in the host response to endotoxin (see section 1:3).

1:5:3. The Potential of Anti-Endotoxin Antibodies for the Therapy of Septicaemia.

A great deal of interest has been generated in the potential of immunoglobulins for the treatment of Gram-negative septicaemia. Successful protection has been observed in some instances and this may therefore provide an appropriate therapeutic agent (for recent reviews see Baumgartner & Glauser 1987b; Cohen 1986; Schedel 1988; Telzak & Wolff 1985; Young 1984; Young 1985c).

Generally speaking, there are two groups of antibodies which exist against the bacterial cell: type-specific antibodies and cross-reactive antibodies. Much of the research with either group of antibodies has been directed towards vaccination for the production

of either hyper-immune globulin or of monoclonal antibodies, and both methods have received much attention.

Antibodies which are type-specific or predominantly type-specific (both monoclonal and hyper-immune) which are directed against the O-antigen of a bacterial cell have been proven to possess protective activities against the organisms to which they are specific, in a variety of animal models (Antonacci *et al* 1984; Barclay *et al* 1986; Colwell *et al* 1984; Coughlin & Bogard 1987; Dunn *et al* 1985b; Dunn *et al* 1988; Griesman *et al* 1973; Greisman *et al* 1979; Johns *et al* 1983; Kim *et al* 1985; Kim *et al* 1988; Kirkland & Ziegler 1984; Munford & Dietschy 1985; McCabe *et al* 1973; Pluschke & Achtman 1985; Sawada *et al* 1984; Sawada *et al* 1985b; Stoll *et al* 1985; Vuopio-Varkila 1988; Young 1984; Zweerink *et al* 1988b) and in human patients (Jones *et al* 1981; Zinner and McCabe 1976). Protective action has additionally been shown through active immunisation of animals, which results in high levels of anti-O-antigen antibodies (Cryz *et al* 1984; Cryz *et al* 1985; Young 1972; Ziegler *et al* 1973). The action of these antibodies is due to enhancement of phagocytosis and bacteriolysis by complement thereby removing organisms from the circulation, and this protective activity of anti-LPS antibodies has been demonstrated even with encapsulated strains of bacteria (Kauffmann *et al* 1986).

The bacteriolytic activity of O-specific antibodies may, however, produce further problems as it results in release of endotoxin, which could then express toxic activities resulting in activation of mediators of septic shock. In addition, a wide range of O-serotypes

and species of Gram-negative bacteria are observed as causes of septicaemia. Thus despite their obvious protective capabilities, these type-specific antibodies have limited therapeutic value. It is possible that a "cocktail" of these antibodies against the most common species and serotypes may provide effective therapeutic activity. The combination of several antibodies with different specificities may, however, result in dilution of antibody against a particular organism and therefore reduction of efficacy. Alternatively, combination of these with an anti-endotoxic agent may prove to be effective.

Furthermore, in many instances, symptoms of septicaemia result from release of endogenous endotoxin, the bacterial source of which cannot be identified. Also, all of the clinical signs of septicaemia may be observed in the absence of an organism being isolated from the blood. Therefore type-specific antibodies to O-antigen (which are bacteriolytic or opsonic) would be of little use because of the diversity of organisms or other possible sources of endotoxin in septicaemia.

It would thus be advantageous to produce an immunoglobulin preparation capable of providing protection against a wide range of Gram-negative organisms, and which enables removal of endotoxin and neutralisation of LPS toxicity. This requires direction of activities towards the cross-reactive region of the lipopolysaccharide molecule - the core glycolipid (CGL).

The core glycolipid region represents a highly structurally and

antigenically conserved component of LPS as discussed in section 1:2. It is therefore possible that antibodies directed predominantly against core region would provide protection from the lethal effects of septicaemia.

The potential of anti-CGL antibodies as protective agents may be queried because of the nature of the LPS molecule _in vivo_ (see section 1:3). In the intact cell the core and lipid A are believed to be hidden by O-antigen chains which may prevent access of the antibody molecule to CGL or other conserved components. It has, though, been demonstrated that full core (R-LPS) or core plus one O-antigen unit (SR-LPS) can be found on cells, thus anti-CGL antibodies may be able to bind to intact cells but they appear to possess only limited opsonic or bacteriolytic activities (see section 1:3). This problem assumes lesser significance when it is considered that endotoxic activities of LPS are expressed only upon release from the cell surface by the means discussed above. This also results in exposure of the core-glycolipid, and means that anti-CGL antibodies can therefore obtain access and bind to the liberated endotoxin. By acting in this manner, anti-CGL antibodies behave as anti-toxins through neutralisation of LPS activation of cells and humoral factors by preventing access of LPS to activation sites, and also by aiding removal of free endotoxin by the RES.

Anti-sera and monoclonal antibodies raised in a variety of animal species against the core glycolipid or to lipid A itself have been shown to be highly cross-reactive in a variety of assay systems, and also effective in protection against the effects of septicaemia

and/or endotoxaemia caused by a wide range of bacteria in many animal models. Protective activity has been demonstrated through prophylactic, therapeutic, or active immunisation (Braude et al 1977; Bruins et al 1977; Coughlin & Bogard 1987; Dunn & Ferguson 1982; Dunn et al 1983b; Dunn et al 1984b; Dunn et al 1985a; Dunn et al 1985c; Dunn et al 1986; Feeley et al 1987; Fenwick et al 1986; Galanos et al 1971; Johns et al 1977; Johns et al 1983; Larrick et al 1987; McCabe 1972; McCabe et al 1973; McCabe et al 1977; Rietschel and Galanos 1977; Sakulramrung and Domingue 1985; Teng et al 1985; Warren et al 1987a; Young & Stevens 1977; Young et al 1975; Young et al 1982).

An extensive study has been carried out by Gaffin and co-workers over a period of several years for the evaluation of a hyper-immune equine plasma (HIEP). This hyper-immune plasma was shown to possess anti-bacterial and endotoxin-binding activities (Wells & Gaffin 1987; Wells et al 1987a) and to be protective in a series of animal models (Gaffin et al 1985b; Gaffin et al 1986; Wells et al 1987b; Zanotti & Gaffin 1985), therefore providing a strong indication of the widely cross-protective activities of antibodies directed to LPS.

Assessment of the protective activity of passively administered anti-CGL antisera has been determined in human septic shock patients. Of particular note is the long series of studies, firstly in animals with rabbit antiserum (Braude et al 1973; Braude et al 1977; Davis et al 1978; Ziegler et al 1973b) then in animals with human antiserum (Ziegler et al 1973a; Ziegler et al 1975), and

finally in human patients with the same human antiserum (McCutchan *et al* 1979; McCutchan & Ziegler 1983; Ziegler *et al* 1978; Ziegler *et al* 1979). This culminated in a study by Ziegler *et al* (1982) for which antiserum was prepared by pooling antisera from human volunteers who had been vaccinated with whole heat-killed *E. coli* J5 cells. Antiserum was administered to patients at risk of septicaemia, and, in comparison to a control group (which received no antiserum) a significant degree of protection against mortality was obtained in patients with bacteraemia (22% in anti-J5 treated patients versus 39% in pre-immune serum treated controls, p=0.011), and in patients with profound shock (44% versus 77% mortality, p=0.003). A further clinical trial with this antiserum was carried out, and protective activity was confirmed (Baumgartner *et al* 1985), and the protective factor was proven to be anti-CGL antibodies (Baumgartner *et al* 1987c). Further evaluation of the protective activity of an anti-*E. coli* J5 antiserum was carried out by Pollack *et al* 1983, with a similar protective activity being obtained.

Some studies for the evaluation of the protective capacity of anti-CGL antibodies have not, however, been able to show protective activities in certain *in vitro* and *in vivo* experimental systems, despite the presence of antibodies directed towards core-glycolipid (Gigliotti and Shenep 1985; Griesman & Johnston 1988; Griesman *et al* 1978; Greisman *et al* 1979; Ng *etal* 1976; Trautmann and Hahn 1985; Vuopio-Varkila 1988). This shows that antibodies (particularly monoclonal antibodies) which recognise conserved regions of the LPS molecule do not all possess protective activities, but that many of those which have been produced do possess these activities.

Therefore it is likely that anti-core preparations could provide a widely protective therapy for septicaemia.

Further evidence for the role of anti-CGL antibodies in protection against Gram-negative septicaemia has been obtained by the observation that the presence of high levels of antibodies prior to infection results in a lower incidence of bacteraemia and an improved survival rate in patients who do acquire bacteraemia (Freeman & Gould 1985a; Freeman & Gould 1985b; McCabe et al 1972; Peter et al 1979; Pollack & Young 1979; Pollack et al 1983; Zinner & McCabe 1976) - see also section 1:3.

One approach which has only recently been considered as a route of obtaining protective antibodies to LPS is that of naturally hyper-immune blood donors (Gaffin 1983; Rivat-Peran et al 1983; Schedel 1985). Screening of blood donor populations has shown that a wide range of antibody levels to a variety of smooth and rough lipopolysaccharides and lipid A molecules exists (see section 1:4). Since it has also been determined that the presence of natural antibody to endotoxin prior to septicaemia correlates with a better rate of survival from septic shock (see above), it is highly probable that appropriately selected donors with high levels of antibodies to inner core and/or lipid A could provide a readily available source of protective antibodies for use in the treatment of septicaemia.

Pooled human sera or IgG fractions which have not been selected for high levels of anti-LPS have been shown to possess protective

activity against septicaemia and endotoxaemia caused by a wide range of organisms in animal models (Abdelnoor et al 1982; Bulay et al 1986; Collins & Roby 1983; Collins et al 1986; Collins et al 1987; Duswald et al 1980; Emerson et al 1986; Harper et al 1987; Hill & Bathras 1986; Iwata et al 1987; Peterson et al 1987; Stephan et al 1985; Stoll et al 1987; Stuttman et al 1987), and in patients, reduction in the incidence of infections was obtained (Duswald et al 1980) although, in another study, no reduction in sepsis or mortality was obtained (Glinz et al 1985). It would therefore be expected that selected high titre donor sera or IgG prepared from these sera might be a highly effective therapeutic agent for the prophylaxis and treatment of Gram-negative septicaemia.

The protective capability of normal human sera with high levels of cross-reactive anti-LPS antibodies has been demonstrated. Sera were assayed for the presence of antibodies to 12 smooth LPS molecules, and those with 40ug IgG per ml or higher were pooled and fractionated. This preparation was shown to contain both type-specific (anti-O-antigen) antibodies, and cross-reactive (anti-CGL) antibodies (Gaffin et al 1985). The pooled IgG was shown to possess antibacterial activity in vitro (Pudifin et al 1985), and protective activity in animal models (Gaffin et al 1981; Gaffin et al 1985a), and was assessed in human septic shock patients (Lachman et al 1984a; Lachman et al 1984b). Results of this trial showed fatality in 40.0% of control patients and in 4.3% of patients receiving immunoglobulin (p=0.00236). This therefore provides a highly effective preparation for treatment of septic shock resulting from septicaemia and endotoxaemia.

Further clinical trials have been carried out with donor sera selected for high titre anti-lipid A antibodies, and preliminary results have shown an improved outcome (Jaspers *et al* 1987; Marget *et al* 1985). One other trial is under way in which human sera possessing high levels of anti-core antibodies are being assessed for their protective capacity, and, once again, preliminary results are indicative of protection (Schedel 1988).

Antibodies against core glycolipid (CGL) obtained from selected blood donors with high levels of antibodies would, therefore, appear to provide a readily available source of antibodies for the treatment of septicaemia, as it avoids the process of vaccination to produce hyper-immune sera (which often produces levels of antibody no higher than found naturally and also produces unpleasant side-effects in vaccinees - Rivat-Peran *et al* 1985; Ziegler *et al* 1982). The production of monoclonal antibodies against conserved epitopes of LPS is another possibility, but this is a time-consuming process which does not necessarily result in protective antibodies. Assessment of antibodies from blood donors is therefore an essential process in the determination of strategies for prevention and treatment of septicaemia.

From the above evidence it appears that passively administered normal human sera, IgG fractions of sera, or monoclonal antibodies selected for a high titre against CGL or lipid A could be an effective means of treatment and prophylaxis of septic shock caused

by Gram-negative septicaemia or endotoxaemia. Efforts to produce a cross-protective immunoglobulin preparation therefore appear hopeful.

The most effective therapy, however, may include elements and agents from several current or developing strategies. The essential element of any treatment is to eliminate endotoxin, thereby removing the progenitor of septicaemia from the circulation and from organ stores. It can, however, be seen that anti-endotoxin antibodies are undoubtedly one of the factors with greatest influence of protection from the toxic activities of LPS.

Objectives of the Current Study.

In view of the complexity of the factors involved in septicaemia and endotoxaemia, this thesis aims to explore several different, though interlinked, aspects of the potential of obtaining a therapeutic IgG preparation from the blood donor population.

1. Levels of anti-core-glycolipid immunoglobulins are assessed in an ELISA system which used as antigens complexes of four rough lipopolysaccharides with polymyxin. The antigenic relationship between lipopolysaccharide present in LPS-polymyxin complexes and other forms of LPS (uncomplexed, outer membrane-bound, and cell-bound) will be assessed as will the levels of IgG to a wider range of LPS molecules to determine the relevance of this assay system for the detection of anti-LPS and in particular anti-CGL antibodies.

2. The alteration of anti-LPS antibodies in patients in septic shock has not been determined out in detail so far. It was thus decided that an assessment was required to determine the presence or absence of any relationship between particular anti-CGL antibodies with levels of endotoxin in these patients.

3. The immunoglobulin response to LPS has not previously been studied in detail. Immunisation of rabbits with a range of Gram-negative bacteria was carried out to determine the immunoglobulin response to LPS from different species and genera and additionally to provide information upon the antigenic relationships between epitopes present on a variety S-LPS and R-LPS and lipid A molecules.

4. The availability of CGL epitopes on intact bacteria has been in

doubt for some time. There was thus a requirement to determine the extent of binding of anti-core and anti-O-antigen monoclonal antibodies to an organism of relevance to septicaemia.

5. It has been determined that hyperimmune globulin produced by vaccination or monoclonal antibodies possessing strong reactivity with CGL is effective in prevention of fatalities arising from septic shock. Little evaluation has been made of the protective capability of normal human serum selected for high levels of IgG to CGL epitopes. Assessment must be made _in vitro_ and _in vivo_ of the anti-endotoxin and anti-bacterial capabilities of human sera and IgG possessing various levels of anti-CGL antibodies both _in vitro_ and _in vivo_.

The results of these studies could therefore provide an indication of the usefulness of selected human immunoglobulin products for the treatment of septicaemia. Additionally, other points of contention currently existing regarding the antigenic presentation of LPS and structural and antigenic relationships between lipopolysaccharides will be investigated. Particular regard will be paid to antigenic relationships between organisms which commonly cause septicaemia with relevance to development of immunotherapeutic strategies.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains used included Gram-negative aerobic and facultative anaerobic rod shaped bacteria with rough or smooth lipopolysaccharide phenotypes. These organisms were obtained from a range of sources as mentioned below. When received, all strains were immediatelly cultured to prepare lyophilised stock cultures.

	ORGANISM	STRAIN		SOURCE
1.	S. typhimurium	R1542	Ra	
2.	" " "	R119	Rb	Dr I.W. Sutherland,
3.	" " "	R878	Rc	Deptartment of Microbiology,
4.	" " "	R1032	Rd	University of Edinburgh,
5.	" " "	R1102	Re	Edinburgh, Scotland.
6.	S. minnesota	R595	Re	
				Dr. G Schmidt,
7.	S. minnesota	R60	Ra	Forschungs Institut,
8.	" " "	R345	Rb	Borstel,
9.	" " "	R5	Rc	Institut fur Experimentalle
10.	" " "	R7	Rd	Biologie und Medizin,
				West Germany.
11.	K. aerogenes	10B	Rb	Dr. I. R. Poxton,
				Deptartment of Bacteriology,
				University of Edinburgh,
				Edinburgh, Scotland.
12.	P. aeruginosa	PAC605		Prof. P. Meadow
				University College,
				London, England.
13.	" " "	Habs type 1		Dr. R. Jones,
				MRC Microbial Pathogenicity Group,
				University of Liverpool,
				Liverpool,
14.	E. coli	J5	Rc	England.
15.	" " "	R1	Ra	Dr. N. Carlin,
16.	" " "	R2	Ra	National Bacteriology Laboratory,
17.	" " "	R3	Ra	Karolinska Institute,
18.	" " "	R4	Ra	Stockholm,
19.	" " "	K12	Ra	Sweden.
20.	" " "	c62	Ra	
21.	" " "	O86a		Prof. I. Ledingham,
				Western General Hospital,
				Glasgow.

22.	*E. coli*	O18;K1	
23.	" " "	O18;K-	
24.	" " "	O16;K1	
25.	" " "	O6;K5	Dr. A. Cross,
26.	" " "	O4;K?	Department of Bacterial Diseases,
27.	" " "	O12;K?	Division of Communicable Diseases,
28.	" " "	O15;K?	Walter Reed Army Institute of Research,
29.	" " "	O8;K?	Washington D.C.,
30.	" " "	O2;K?	USA.
31.	" " "	O1;K?	
32.	" " "	O8;K?	
33.	" " "	O75;K?	

Lipopolysaccharides.

LPS was prepared from smooth organisms by the hot phenol-water method of Westphal *et al* (1952) and from rough mutant bacteria by the phenol/chloroform/petroleum method of Galanos *et al* (1969) (for both methods see below). Additional LPS was obtained from List Biologicals Laboratories, Campbell, California, USA:

E. coli O111:B4	Product no. 201
" " " K12 mm294 (complete core)	Product no. 303
" " " K12 D31m4 (Re core)	Product no. 302
" " " K12 D31m4 (lipid A)	Product no. 402
S. minnesota wild type	Product no. 220
" " " R60 (Ra core)	Product no. 312
" " " R345 (Rb core)	Product no. 310
" " " R5 (Rc core)	Product no. 308
" " " R7 (Rd core)	Product no. 306
" " " R595 (Re core)	Product no. 304
" " " lipid A	Product no. 401

Sera and Monoclonal Antibodies.

Samples of human sera were obtained from blood donors at the South East Scotland National Blood Transfusion Centre in Edinburgh. Sera were screened after being held at 4°C overnight. Sera were then stored at -40°C and thawed at 4°C overnight for subsequent assay.

Two monoclonal antibodies, one with high specificity for the O-antigen of *E. coli* O18:K1 (McAb SZ185/2.5.5), and the other with

high specificity for the outer core region of E. coli O18:K1 (McAb SZ27/150.3) were obtained from fusions carried out in the Department of Surgery, University of Edinburgh Medical School.

Purified IgG prepared from blood donors with high titres (Ps+) and low titres (Ps-) of antibodies to a P. aeruginosa vaccine, and IgG from volunteers vaccinated with this vaccine were obtained from the Scottisn National Blood Transfusion Service Protein Fractionation Centre, Edinburgh.

Selected donor sera were used for more detailed analyses. These were GL+, possessing high levels of anti-CGL and given an arbitrary value of 100%; GL-, possessing very low levels of anti-CGL (less than 10% in comparison to GL+); HI-NS, containing a very high titre of antiCGL (602% in relation to GL+); and MED1 and MED2 with anti-CGL values of 60% and 110% respectively in comparison to GL+.

Septic Shock Patients.

Six patients showing symptoms of septic shock from the Intensive Therapy Unit, Department of Surgery, Western Infirmary, Glasgow were available for assay. Blood samples were obtained daily, from which serum was separated. Sera were assayed in ELISA and by Limulus amoebocyte lysate assay (see below).

Preparation of IgG from Human Sera.

IgG fractions were prepared from sera in a two-step process by precipitation and ion-exchange chromatography. Immunoglobulin was precipitated by the addition of crystalline ammonium sulphate (Sigma) to serum to give 50% saturation (equivalent to a final concentration of 0.313g/ml). The $(NH_4)_2SO_4$ was allowed to dissolve

with stirring. The precipitate was harvested by centrifugation (MSE Hi-Spin) at 10,000g for 60min. The pellet was resuspended in a small volume of 10mM potassium hydrogen-phosphate ($K_2HPO_4.3H_2O$ - Sigma), pH 6.8. After being dissolved, the solution was adjusted to its original volume with 10mM $K_2HPO_4.3H_2O$, pH 6.8. This was re-precipitated, recentrifuged and resuspended as above. Ammonium sulphate was removed by ultrafiltration.

Ultrafiltration was carried out in Collodion bags (Cat. No. SM 13200E, Sartorius GmbH, Gottingen, West Germany) with a molecular weight cut-off of 12,400, in a Collodion bag holder (Cat. No. SM 16305, Sartorius GmbH, Gottingen, West Germany) under a vacuum of 50mm Hg, with 3 volumes of 10mM $K_2HPO_4.3H_2O$. Once ultrafiltration was complete, samples were adjusted to their original volume.

The second step in the purification process involved the use of ion exchange DEAE-cellulose (Whatman DE52). The required volume of pre-swollen gel was prepared as a slurry by suspending in 6ml of 10mM $K_2HPO_4.3H_2O$ pH 6.8, for every 1.0g of DEAE-cellulose. The cellulose was allowed to settle and the excess buffer was decanted. These steps were carried out repeatedly until decanted buffer was also at pH6.8. The slurry was finally allowed to settle and excess buffer was removed until a final volume of 20% over the volume of slurry was obtained. This slurry was used to prepare a column (35mm x 315mm). Buffer was passed through the column at a constant flow rate (45ml per h per cm^2 of column cross-sectional area) until the column bed height was constant. The column was then equilibrated until the pH of the eluent was the same as the loading buffer (10mM $K_2HPO_4.3H_2O$, pH 6.8). The ammonium sulphate precipitate was then passed through the column and elution of IgG was monitored by

observing a peak of absorbance at 280nm. Other immunoglobulins and serum proteins were eluted by passing 1.4M NaCl in potassium phosphate through the column. Elution was again monitored by measuring absorbance at 280nm. The column was regenerated by washing the column through with $K_2HPO_4.3H_2O$ buffer, pH6.8.
Purified IgG was then concentrated to its original volume by vacuum dialysis using the ultrafiltration system previously described.

Culture of Bacteria.

All bacteria were cultured at 37°C in an orbital incubator (Cat. No. IH-465, Gallenkamp, Widnes, Lancashire, UK). Starter cultures were prepared by inoculation from blood agar - BA - stocks which were originally prepared from freeze-dried stocks. Bacteria were transferred onto fresh BA monthly for a total of four subcultures, after which time fresh cultures were prepared from freeze-dried stocks.
Bacteria were inoculated into 10ml nutrient broth to prepare a starter culture, which could then be inoculated into larger volumes for further growth. Purity of cultures was determined by Gram-staining and by plating onto blood agar.

Culture Media.

a) Nutrient broth.

Gibco nutrient broth was prepared at the Blood Transfusion Service Protein Fractionation Centre. This culture medium was sterilised by ultrafiltration.

b) Minimal medium.

MALKA minimal medium was prepared as a modification of the medium of

Robert-Gero et al 1970. Stock solutions were prepared as follows.

Solution A: 73.4 mg/ml Na_2HPO_4, 32.4mg/ml KH_2PO_4, pH 7.2.

Solution B: 20.5 mg/ml $MgSO_4.7H_2O$.

Solution C:20% w/v glucose.

Solution D: 1.83 mg/ml $FeSO_4.7H_2O$ in sterile distilled water, to which 1 drop of concentrated HCl or H_2SO_4 was added.

Solution E: 50.0 mg/ml $(NH_4)_2SO_4$.

All solutions were prepared with sterile distilled water and were filter sterilised. Solutions A and B were stored over $CHCl_3$. Solution C was filter sterilised. Solution D was not to be autoclaved.

To prepare 1000ml of MALKA, 20ml A, 20ml B, 20ml C, 1ml D, and 20ml E were added 919ml of distilled water.

Modifications of this medium were also used in which 10%, 1%, and 0% of the concentration of magnesium salt (solution B) were present.

c) Nitrogen Deficient Medium (NDM).

This was prepared following the method of Sutherland & Wilkinson (1965), and contained the following: 1g yeast extract (Oxoid); 1g casamino acids (Difco technical grade); 10g Na_2HPO_4; 3g KH_2PO_4; 0.2g $MgSO_4.7H_2O$; 1g K_2SO_4; 1g NaCl; 0.01g $CaCl_2$; and 0.01g $FeSO_4$. This solution was made up to 1000ml with distilled water and autoclaved. To this, 20% w/v filter-sterilised glucose solution was added to give a final concentration of 2% glucose w/v.

d)Trypticase Soy Broth - TSB.

Trypticase soy broth (Prod. No. 152-4980, Gibco Ltd., Paisley, Scotland) was prepared at 30g/1, to which 1.5% w/v nutrient agar (Prod. No. 152-3560, Gibco Ltd., Paisley, Scotland) was added.

e) Sheep serum.

This was obtained from Moredun Animal Research Institute, Gilmerton Road, Edinburgh. After preparation, serum was filter-sterilised (0.45um pore size) and stored at -20°C. Samples were thawed immediately prior to use.

Bacterial Counts.

Viable counts were performed by a modification of the Miles-Misra method. Serial dilutions of bacteria were prepared in sterile PBS, and five 20ul volumes were spotted onto BA. Viable count was calculated from the number of colonies produced after overnight incubation at 37°C.

Counting of whole cells was performed in a haemocytometer (Improved Neubauer, depth 0.1mm, $1/400mm^2$, Weber & Sons, Lancing, England).

Chemical Assays.

Assays for carbohydrate and protein were carried out by the methods of DuBois *et al* (1956) with glucose standard and Lowry *et al* (1951) with bovine serum albumin standard respectively.

Preparation of Smooth Lipopolysaccharides.

LPS from organisms of smooth phenotype were prepared by the method of Westphal *et al* (1952). Batches of bacteria were cultured overnight in nutrient broth as described above. Volumes (8 to 12 times 1000ml) were grown overnight then harvested and washed twice in phosphate-buffered saline - PBS (0.05*M* sodium phosphate, 0.15*M* sodium chloride, pH7.4). The bacterial pellet was freeze-dried and weighed. The dried bacterial pellet was resuspended in distilled

water (dw) to a concentration of 5% w/v, and heated to 65-68°C in a water bath. To this an equal volume of 90% (w/v) phenol (BDH Analar - 90g heated to 45°C then made up to 100ml with dw) in distilled water, also at 65-68°C, was added and the mixture was stirred for 15 minutes. The mixture was then cooled in an iced water bath to separate the phenol and aqueous phases. Centrifugation at 5000-10000g for 15 min was applied separate further the two phases. From this, the upper aqueous layer was removed and the extraction was repeated on the lower phenol phase. The aqueous phases from both procedures were pooled and cleared if required (10000g, 15 min) then dialysed overnight (or until phenol was no longer detectable) against running tap water. After dialysis the sample was cleared again if required, reduced to approximately 20% of its original volume in a rotary evaporator, then ultracentrifuged for 3h at 100000g. This produced a pellet which was resuspended in distilled water and subjected to further centrifugation. The final pellet was resuspended in a small volume of distilled water, freeze-dried, then weighed. This LPS was stored at -20°C.

Preparation of Rough Lipopolysaccharides.

The petroleum ether/chloroform/phenol (PCP) method of Galanos *et al* (1969) was used to prepare LPS from rough mutant bacteria. This required a stock extraction solution containing petroleum ether - boiling point 40°-60°C (BDH Analar), chloroform (BDH Analar), and 90% phenol (see above) in the ratio 8:5:2 by volume.

Bacteria from an overnight culture were harvested and washed twice in distilled water, then lyophilised and weighed. Dried bacteria were resuspended in PCP to 25% w/v, homogenised for 2 min below

20°C, then centrifuged at 10000*g* for 15min. The supernate was filtered (Whatman No.1) into a round bottom flask. The pellet was re-extracted by the same procedure and the filtered supernates were pooled. The supernate was rotary evaporated to remove the petroleum spirit and chloroform. LPS was precipitated by the addition of distilled water in a drop-wise manner (approximately 0.4ml was required). Once precipitate formed, the solution was left to stand for 10min. This mixture was centrifuged at 5000*g* (Heraeus Christ, Bactifuge) for 10 min to produce a solid pellet. The supernate was discarded and, after draining the tubes, the pellet was washed in 80% phenol three times, recovery being obtained by centrifugation. The final pellet was washed twice in ether and dried *in vacuo*. Once dry, the pellet of crude LPS was resuspended in 3-5ml of dw, then sedimented by centrifugation at 100000*g* for 4h. The final LPS pellet was dissolved in dw and lyophilised.

An alternative means of precipitation was required for some of the R-LPS preparations - particularly *P. aeruginosa* R-LPS - as dw was found to produce an inadequate yield. Qureshi *et al* (1982) described an improved precipitation by addition of six volumes of diethyl ether/acetone (both analytical grade in the ratio of 1:5 by volume) to one volume of phenol solution. After addition of diethyl ether/acetone the mixture was left to stand for 60min to allow maximal precipitation. The precipitate was then washed and centrifuged as described

Preparation of Outer Membrane Complex.

This was prepared by a modification of the method of Poxton (1979). An overnight culture of bacteria was harvested and washed twice in PBS by centrifugation at 12000*g* for 10min. The bacterial pellet was

resuspended in 1/50th of the initial volume in PBS containing 10mM EDTA (ethylene diamine tetra acetic acid disodium salt, BDH Analar), and incubated at 45°C for 30-40min. The suspension was then sonicated for 50sec in an ultrasonic bath (Model 6441A, Dawe Instruments Ltd., Western Avenue, London W3 OSD, UK) and the unbroken cells were removed by repeated centrifugation until no cells were visible in the supernate under phase contrast microscopy. The supernate contained outer membrane which was assayed for carbohydrate and protein content.

Proteinase K Digestion of Bacteria.

Proteinase K (Sigma) digestion (Hitchcock & Brown 1983) of both smooth and rough bacteria was performed to prepare lipopolysaccharides for analysis by PAGE and immunoblotting. Bacteria were harvested, washed twice in PBS, then resuspended in sterile PBS to give an absorbance of between 0.5 and 0.6 at 525nm as measured in a spectrophotometer. This suspension (1.5ml) was harvested (3min 1000g Beckman Microfuge B) in a 1500ul Eppendorf tube (Elkay) and the pellet was suspended in 50ul single strength PAGE sample buffer (see below). This suspension was boiled for 10min. Once cooled, 10ul of sample buffer containing 25ug of proteinase K was added (2.5mg/ml in sample buffer), followed by incubation in a 60°C water bath for 60min. Samples were stored at -20°C until required. Samples (10ul) were loaded onto polyacrylamide gels for electrophoresis.

Polyacrylamide Gel Electrophoresis - PAGE.

Polyacrylamide gel electrophoresis was carried out with SDS-free gel

buffers following the method of Pyle & Schill (1986), which itself is an adaptation of the method of Laemmli (1970). Separating gel contained 17.5ml separating gel buffer (0.75M Tris-HCL pH8.8 - BDH Analar), 3.5ml dw, and 12.25ml 40% acrylamide stock solution (40g acrylamide - BDH electrophoresis grade - plus 1.08g methylene-bis-acrylamide - BDH electrophoresis grade - made up to 100ml with dw). This mixture was de-aerated under vacuum after which 1.75ml ammonium persulphate ($(NH_4)_2S_2O_8$ BDH Analar - 15mg/ml) and 50ul TEMED (NNN'N'-tetramethyl-1,2-diaminoethane - BDH) were added. This was prepared as a 160mm x 125mm x 1.5mm slab gel, and was overlayed with water saturated butan-2-ol until set. After removal of butanol the stacking gel was poured. This contained 5.0ml stacking gel buffer (0.25M Tris-HCl pH6.8), 3.5ml dw, and 1.0ml 40% acrylamide stock solution. After de-aeration, 0.5ml ammonium persulphate solution and 20ul TEMED were added. The solution was poured onto the separating gel and a comb inserted.

The electrode buffer consisted of 0.025M Tris (BDH Analar), 0.192M glycine (BDH specially pure), pH8.3. To this 0.1% by weight SDS (sodium dodecyl sulphate - Fisons "Primar" grade) was added.

Samples were prepared in single strength sample buffer which contained 0.0625M Tris pH6.8 (BDH Analar) in which 2% SDS by weight, 10% glycerol (Fisons A.R.) by volume, 1% 2-mercaptoethanol by volume, and 0.001% bromophenol blue by weight were present. Lipopolysaccharide and outer membrane preparations were dissolved in buffer and boiled for 3min prior to loading onto gels. Proteinase K digestions were loaded directly onto gels.

Samples were electrophoresed through the stacking gel at a constant 60V, and through the separating gel at a constant 150V until the

front had run 75mm. After electrophoresis, samples could be analysed by staining for lipopolysaccharide or protein or by immunoblotting after transferrence to nitrocellulose.

Staining PAG for Lipopolysaccharide.

An adaptation of the methods of Tsai & Frasch (1982) and Hitchcock & Brown (1986) was used to visualise LPS in polyacrylamide gels - PAG. After electrophoresis, the gel was placed in fixative (7% acetic acid - BDH Analar- and 25% isopropanol - BDH General Purpose - by volume in dw) overnight. Once fixed, the fixative was poured off and the gel was oxidised for 5 min in a solution of 1.05g periodic acid (H_5IO_6 - BDH General purpose) in 150ml dw to which 4ml of fixative was added. The PAG was then washed for 4h in dw with at least four changes of water. Silver staining was carried out by placing the PAG in 100ml of staining solution which contained 21ml 0.36% NaOH, 1.4ml ammonium solution, 4.0ml $AgNO_3$, and 73.6ml dw. After staining for 10min the gel was washed again for 40min with four changes of dw. Lipopolysaccharide was visualised by the addition of 0.005g citric acid (Fisons A.R.) dissolved in 100ml 0.019% formalin (prepared by dilution of BDH Analar formaldehyde solution. Once colour had developed sufficiently, the reaction was stopped by washing the gel in dw 3 to 4 times. The gel was then stored in the dark.

Electrophoretic Transfer of Antigens from PAG to Nitrocellulose.

Lipopolysaccharides and other antigens which had been subjected to PAGE were transferred to nitrocellulose (0.2um pore size) for immunochemical analysis. A modification of the method of Towbin (1979) was used as follows: the gel was removed from its cassette

and a nitrocellulose sheet soaked in electrode buffer (12g Tris - BDH General purpose, 57.68g glycine, 1000ml methanol - BDH Analar, and 4000ml dw - pH8.3) was placed on top. The gel and nitrocellulose were then sandwiched between two Scotchbrite^{TM} pads in a cassette and the assembly was placed into a gel tank containing electrode buffer. The gel was placed towards the cathode and the nitrocellulose towards the anode. A constant current of 40mA was applied overnight at 4°C.

Immunological Staining of Antigens.

After transfer to NC antigenic reactivity was examined. Sheets of NC were firstly washed in Tris-buffered saline - TBS (4.84g Tris, 58.48g NaCl, 2000ml dw - pH7.5) for 10min. At this point NC could be dried and 2ul volumes of antigen dotted on to act as controls. Dots were allowed to dry then the sheet was placed in blocking solution (TBS containing 3% w/v gelatin - Bio-Rad EIA purity grade) for 35-40min. Once blocked, the NC sheet was transferred into antibody buffer (TBS with 1% w/v gelatin) containing an appropriate dilution of antibody, and incubated for 3h at room temperature. After reaction with antibody solution, the sheet was washed in TTBS (TBS with 0.025% Tween 20 v/v - polyoxyethylenesorbitan monolaurate, Bio-Rad EIA Purity grade) for 15min then reacted with secondary antibody. This was either anti-human or anti-mouse Ig Horse radish peroxidase (HRP) conjugate depending upon the species of the first antibody. TTBS was again used to wash the NC for a further period of 15min and then the binding of antibody was visualised by addition of 30mg HRP colour reagent (Bio-Rad EIA Purity grade) dissolved in 10ml ice cold methanol added to 50ml TBS containing 30ul H_2O_2. The

development of colour was stopped after 30min by washing the NC in dw. The blot was dryed and stored in the dark.

An avidin-biotin adaptation of this method was also carried out. In this case the secondary antibody was replaced with biotinilated anti-human IgG (E.Y. diluted 1:1000) which was incubated for 60min. Nitrocellulose was then washed twice with TTBS for 10min each, then avidin-Horse radish peroxidase conjugate at a dilution of 1:1000 was incubated with the blots for 30min. Sheets were washed again in TTBS (2 x 10mins) and rinsed in TBS. Colour development was carried out as above.

A dot blot system was also assessed. In this biotin labelled anti-human IgG from two sources (E.Y. and Vector) and avidin labelled Horse radish peroxidase from two sources (BRL and Vector) were used as probes after reaction of antigens (2ul of a solution containing 10mg/ml of LPS) with primary antibody.

Alkaline phosphatase labelled secondary antibody was also used in some instances. All stages were the same except for colour development reaction. After washing in TTBS, the NC was rinsed in substrate buffer (0.06M sodium borate pH9.7 with 1.2mg/ml $MgSO_4.H_2O$). This was replaced with substrate buffer containing 0.25mg/ml o-dianisidine tetrazotized and B-naphthyl acid phosphate (monosodium salt), both from Sigma. Once developed for 15min the blot was washed for 15min in methanol/acetic acid/dw (5:1:5 by volume) then washed in dw.

Coating of ELISA Microtitre Strips.

Coating of microtitre strips was carried out following a modification of the method of Barclay & Scott (1987). Mini-sorb tubes (Nunc Inter Med, Kamstrup, Roskilde, Denmark) were used throughout.

a) Coating with LPS-polymyxin complex.

Stock solutions of both polymyxin and LPS were sonicated prior to mixing. Complexes were formed by adding polymyxin B sulphate (Sigma chemicals) at a concentration of 0.2m_M_ with lipopolysaccharide at a concentration of 0.1m_M_ and mixing for 30min at room temperature. This solution was then dialysed in 2000 molecular weight cut-off cellulose tubing (Spectrum Medical Supplies Industries Inc.) overnight against dw to remove excess unbound polymyxin. Complex could be stored at -20°C. Molarity of LPS was calculated from the estimated molecular weights of LPS as determined by Morrison & Jacobs (1976):

LPS TYPE	MOLECULAR WEIGHT
wild type (smooth)	15,000
Ra and Rb type	4,500
Rc type	4,150
Rd and Re type	3,100
lipid A	2,500

To coat strips (Microwell Immuno Quality F8 Medium binding strips, Nunc Inter Med), complex was diluted 1:50 into coating buffer (0.05_M_ carbonate/bicarbonate buffer: 6.2mg/ml $Na_2CO_3.H_2O$, 4.2mg/ml $NaHCO_3$, pH9.6 plus 0.05% sodium azide w/v) and 100ul was added to each well. Strips were incubated overnight at room temperature then washed four times with wash buffer (Oxoid Dulbecco "A" solution, pH7.2, containing 0.05% Tween - Sigma). Wells were post-coated with PBS containing 5% (w/v) bovine serum albumin and 0.05% (w/v) sodium

azide (both Sigma), 100ul of which was added to each well. After overnight incubation at room temperature, strips were washed as described above then stored at -20°C until required.

b) Coating with Purified LPS.

Coating with LPS was carried out by preparation of 0.1m$\underline{M}$ solution of LPS, which was diluted 1:50 in coating buffer (as above), and 100ul was placed into microtitre strip wells. Incubation, washing and post-coating was carried out as described for LPS-polymyxin coating.

c) Coating with Outer Membrane Complex.

This was coated as above. Molarity of LPS in the sample was calculated by comparison of carbohydrate concentrations of the LPS and outer membrane preparations, and determining the volume required to give comparable carbohydrate (and thus LPS) concentrations. The protein concentration of this was also measured.

d) Coating with Bacteria - Viable and Heat-killed.

Bacteria were grown overnight in Gibco nutrient broth, harvested, and washed twice in phosphate-buffered saline. The concentration of bacteria was determined by counting in a haemocytometer, then the suspensions were heated in a boiling water bath for 10min if required. Viability was determined by plating onto blood agar. Carbohydrate concentration was determined as a rough indication for total LPS concentration, and the suspension was diluted in PBS to give approximately 0.1m$\underline{M}$ LPS. Coating of strips was carried out as described above.

Enzyme-Linked Immunosorbent Assay - ELISA.

The microtitre strips were coated for ELISA as described above. Antibody samples were thawed and prepared in dilution buffer (wash

buffer - see above - containing 0.5% (w/v) bovine serum albumin - Miles Laboratories - and 4% (w/v) polyethylene glycol 6000 - Sigma). Dilutions of antibodies were added to plates at 100ul per well. After incubation for 60min at 37°C, the plates were washed 4 times with wash buffer (as above). Secondary antibody (sheep anti-human IgG urease conjugate, rabbit anti-human IgM urease conjugate, goat anti-rabbit IgG urease conjugate or goat anti-rabbit IgM alkaline phosphatase conjugate - Commonwealth Serum Laboratories), prepared in dilution buffer, was added at 100ul per well after plates were dried. Plates were washed as before after 60min incubation with secondary antibody at 37°C. Further washing with dw was carried out, then 100ul urease substrate (Commonwealth Serum Laboratories) was added per well. After development for up to 60min at room temperature, the reaction could be stopped by addition of 20ul 1% w/v thimerosal (Sigma). Absorbance was read in a Titertek Multiscan plate reader. A wavelength of 590nm was required for the urease conjugate.

Absorption of Sera.

Absorption was carried out on fresh and heat-inactivated (56°C, 60min) sera with either viable or heat-killed organisms by the following method: bacteria were suspended to a concentration of approximately 10^8 organisms per ml. Bacterial suspension (400ul) was placed in a 1500ul Eppendorf tube and bacteria were harvested by centrifugation. After removal of PBS, bacteria were resuspended in 400ul serum, then re-centrifuged. This step was repeated three times to ensure maximal absorption of antibodies by bacteria. Samples of absorbed sera were then assayed in ELISA. For EPICS, absorptions

were carried out with a bacterial concentration of 10^9 organisms per millilitre of serum for three absorption steps.

ELISA Competition Assay.

This was carried out by a modification of the above-mentioned ELISA procedure. A doubling dilution series of inhibitor (*S. typhimurium* LPS, LPS-polymyxin complex, or outer membrane complex) was prepared in ELISA dilution buffer. Serum (50ul, diluted 1:50 in dilution buffer) was added to 50ul of each dilution of inhibitor, and mixed. Inhibitor-serum mixtures were incubated for various times at 37°C, then the 100ul was placed onto the appropriate ELISA strips, and ELISA was carried out as described above.

Chromogenic Limulus Amoebocytyte Lysate Assay.

A microtitre plate adaptation of the CoaTest/Endotoxin kit (KabiVitrum Diagnostica UK, Uxbridge, England) chromogenic *Limulus* amoebocyte lysate assay was carried out. All procedures were carried out in a sterile laminar flow cabinet, and Dynatech Immulon A microtitre plates were used throughout. Three different systems were used for measurement of:

a) endotoxin levels in serum,
b) endotoxic activity of purified lipopolysaccharides or IgG,
c) inhibitory activity of serum, IgG, or polymyxin on LPS.

a) Endotoxin Activity in Human Serum.

Serum samples were stored at -20°C, then thawed overnight at 4°C. Serum was diluted 1:10 into PF-dw to give a final volume of 1.0ml in

a Mini-Sorb tube held at 70°C in a heating block, and the diluted serum was held at this temperature for 5min to inactivate LAL inhibitors and activators. After heating, the tubes were cooled by placing into an ice-water bath, at which temperature samples remain stable until assayed. Duplicate serum samples (30ul) were placed in wells in a microtitre plate, one sample being used for determination of *Limulus* amoebocyte lysate activity and the other as a blank. To each test sample, 30ul of PF-dw was added, and to each blank, 90ul PF-dw was added. *Limulus* amoebocyte lysate (LAL) was reconstituted with PF-dw to the recommended concentration, and after standing at room temperature for 10min, 30ul was added to each test sample. These were mixed, and the plates were incubated at 37°C for 40-45min. LAL chromogenic substrate (S-2423) was reconstituted with PF-dw as recommended, then diluted 50:50 with LAL substrate buffer. Buffer-substrate mixture (60ul) was added to each test sample, and plates were incubated for a further 3min at 37°C. The reaction was stopped by the addition of 60ul of 50% v/v acetic acid to test and blank samples, and the absorbance was measured at 405nm. LAL activity was calculated by relation to a standard curve prepared by dilution of the endotoxin supplied (*E. coli* 0111:B4 lipopolysaccharide) in dw.

b) Endotoxic activity of Purified LPS.

LPS was diluted from a stock solution (1mg/ml) with pyrogen-free distilled water (PF-dw). Mini-Sorb tubes were used to avoid adsorbance of LPS onto tube walls. LPS solution (30ul) diluted appropriately was added to 30ul PF-dw in microtitre plate wells. *Limulus* amoebocyte lysate (LAL) was reconstituted with PF-dw as above. LAL (30ul) was added to each microtitre well, and after

mixing, the plates were incubated at 37°C for 25min. LAL substrate was prepared as above, then 60ul was added to each well, and this was incubated for a further 3min at 37°C. The reaction was stopped by the addition of 60ul of 50% v/v acetic acid, and the absorbance was measured at 405nm. LAL activity could be calculated by relation to a standard curve prepared as above.

An adaptation of the method of Piotrowicz and McCartney (1986) was also used. Samples (30ul) were prepared in PF-dw. To samples a reaction mixture (90ul - containing equal parts by volume of LAL, chromogenic substrate and substrate buffer) was added. Colour development could be determined at various time points without requiring the addition of acetic acid to stop colour development.

c) Inhibition of LAL Activity of LPS.

For evaluation of the inhibitory action of serum, purified IgG, or polymyxin on LAL activity, the following procedure was carried out: volumes of the appropriate LPS concentrations (30ul) - prepared as in (b) - were placed in microtitre plate wells. To these, 30ul of inhibitor solution was added, and the solution was mixed and pre-incubated at room temperature if required. To this, 30ul of LAL was added, and incubation was carried out at 37°C for 25min. LAL substrate (60ul) was then added, and after 3min further incubation at 37°C the reaction was stopped by addition of 60ul of 50% v/v acetic acid. Absorbance was measured at 405nm.

Fluorescent Labelling of Bacteria.

E. coli O18:K$^-$ and *E. coli* O18:K1 were grown under a variety of conditions as detailed in RESULTS, and 1.0ml samples were removed and bacteria were harvested (3min, Microfuge B, Beckman) then washed

three times in phosphate buffered saline. Samples were divided into three equal volumes in 1500ul Eppendorf tubes; one as a control, and one for each of two murine monoclonal antibodies (one directed at the core region and one at O-antigen). After centrifugation, two of the bacterial pellets were resuspended in one or other monoclonal antibody (diluted 1:10 in PBS), and incubated at room temperature for 30min. The third sample of bacteria was retained for incubation with FITC-labelled antibody. The suspensions of bacteria in monoclonal antibody solutions were precipitated by centrifugation, and washed three times in PBS. All three samples of bacteria were then resuspended in a 1:40 dilution of FITC labelled anti-mouse IgG (Sigma, $F(ab'_2)$fragment), and incubated for 30min at room temperature. Samples were washed three times in PBS and finally resuspended in saline (0.85% w/v NaCl - BDH Analar - in dw) containing 1% formalin (BDH Analar), in which stability of samples is retained when stored in darkness at 4^oC. Binding of antibodies to the bacterial surface was determined by flow cytometry. For this, a Coulter EPICS "C" flow cytometer was used with a laser output of 500mW at 488nm to excite fluorescence. A flow rate of 1000 bacteria per second was used and 10-40000 cells were analysed. Bacteria were selected by gating on a one parameter histogram measuring forward angle light scatter (FALS), and the percentage of cells showing fluorescence above background levels was calculated.

Longitudinal Immunisation of Rabbits.

A longitudinal immunisation programme in rabbits was carried out. Samples of blood (1ml) were removed from Dutch rabbits at intervals (days -30, -12, and -5) prior to immunisation, and were continued at

7-day intervals after immunisation from days 2 to 163. Rabbits were immunised with 10^8 heat-killed bacteria on days 0, 28, 56, 84, 112, and 140, as detailed in RESULTS. Antibodies (of both IgG and IgM classes) to 31 lipopolysaccharide antigens (10 S-LPS, 19 R-LPS, and 2 lipid A) were assayed in the polymyxin-LPS ELISA system described above with anti-rabbit IgG urease conjugate (Commonwealth Serum Laboratories), and anti-rabbit IgM alkaline phosphatase conjugate (Commonwealth Serum Laboratories). Absorbances were measured at 590nm and 405nm respectively. Serum was removed from the blood sample after clotting and centrifugation (4000g, Heraeus Christ Bactifuge).

Lethality and Protection Studies in Animal Models.

Lethality of purified LPS or viable bacteria was assessed in a variety of mouse strains. All animals were obtained from the Department of Bacteriology, University of Edinburgh animal house where they were bred and where all procedures were carried out. Mice were given food and water ad libitum, and lethality was observed for a period of 3 days.

a) Non-compromised models of endotoxaemia and bacteraemia.

Outbred Swiss white mice (10-12 weeks old), or inbred C57black/6 mice (8-10 weeks old), were challenged intravenously (i.v.) or intraperitoneally (i.p.) with graded doses of LPS dissolved in PBS or dw, or viable bacteria prepared by resuspending a washed overnight culture of bacteria in PBS. Bacterial concentration was calculated by comparison of absorbance of the washed culture with a standard curve of absorbance at 600nm versus viable count, and the appropriate dilution was then calculated. Administration of a

variety of immunoglobulin preparations was carried out i.v. or i.p. as detailed in the RESULTS section.

b) Galactosamine model of endotoxaemia.

Following the method of Galanos et al (1979), inbred C57bl/6 mice (6-8 weeks old) were challenged with LPS plus galactosamine (D(+)galactosamine hydrochloride, Sigma - 8mg per mouse) both dissolved in PBS. LPS and galactosamine were inoculated intraperitoneally either as a mixture, or with the LPS immediately following galactosamine. Once again assessment of immunoglobulins was carried out, as detailed in the RESULTS.

c) Mucin-Haemoglobin model of bacteraemia.

Inbred C57bl/6 mice were sensitized to the lethal effects of bacteria by the co-inoculation of mucin, haemoglobin, and bacteria. Mucin (porcine stomach mucin, type II, crude - Sigma) was prepared as a 4% w/v solution in PBS, and was autoclaved in 50ml samples which were stored at -20°C until required. Haemoglobin (bovine, BDH technical grade) was prepared aseptically as a 16% w/v suspension in PBS, and 25ml volumes were stored at -20°C. Bacterial suspensions were prepared from overnight cultures in Gibco nutrient broth. Bacteria were washed twice in PBS, then resuspended in PBS containing 10% (v/v) glycerol (Fisons A.R.), and stored at -70°C. A viable count was carried out on the bacterial stock solution, and bacteria were diluted in PBS to a dose appropriate for challenge. Mice were challenged i.p. with 0.5ml of a mixture containing mucin at a final concentration of 6.0%, haemoglobin at a concentration of 1.0%, and bacterial suspension (this was prepared by mixing 0.2ml mucin stock solution, 0.125ml haemoglobin solution and 0.175ml bacterial suspension).

Immunoglobulin preparations were assessed for their capacity to protect from lethal challenge of bacteria by i.p. inoculation as detailed in RESULTS.

d) Neutropenic Mouse Model.

Neutropenia was induced in mice by administration of 10mg of cyclophosphamide (Sigma) dissolved in 0.3ml PBS to 6-8 week old C57bl/6 mice. Once neutropenia had developed (48h) mice were challenged with 0.2ml of a mixture of equal parts of bacterial suspension (*E. coli* O16:K1) and haematin (0.244% w/v in sterile PBS). Immediately after bacterial challenge mice were administered with 100ug of purified human immunoglobulin dissolved in sterile PBS (0.2ml) or with PBS.

RESULTS

3:1. Detection of Anti-Lipopolysaccharide Immunoglobulins.

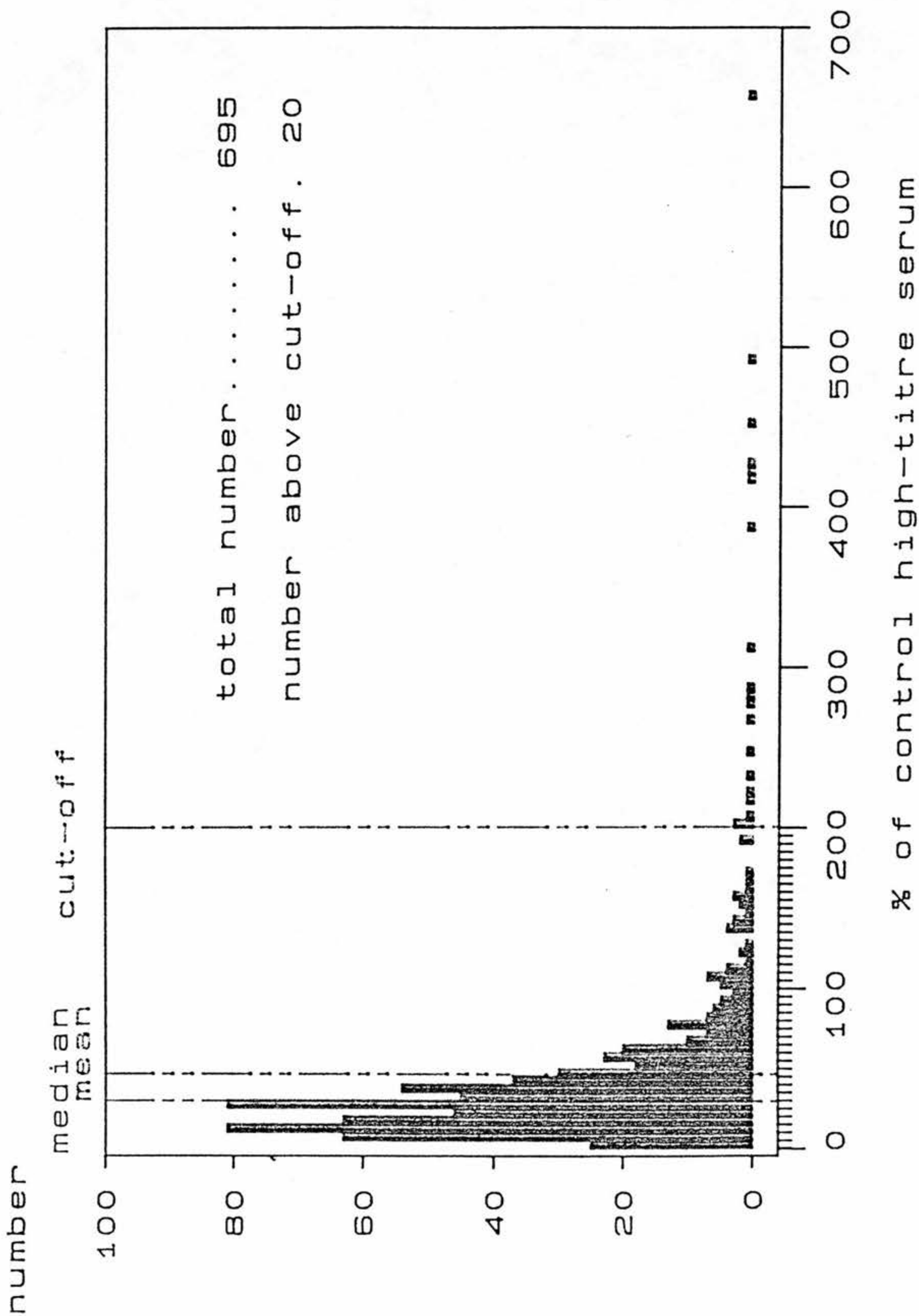
3:1:1. ELISA Survey of Anti-CGL IgG in the Blood Donor Population.

Assay of blood donor sera for anti-LPS core glycolipid IgG was carried out as part of a routine screening procedure at the Scottish National Blood Transfuion Service in Edinburgh. An ELISA system was used in which a "cocktail" containing equimolar amounts of R-LPS from four organisms complexed to polymyxin was used as antigen. LPS from *E. coli* J5 (Rc-type LPS), *S. typhimurium* R878 (Rc), *P. aeruginosa* PAC605 (Rc), and *K. aerogenes* M10B (Rb) were each present at a concentration of 0.025m*M*, giving a total LPS concentration of 0.1m*M*. This "CGL-pool" was used to assay human serum, diluted 1:100 as described in "**MATERIALS AND METHODS**". Absorbance was measured at 590nm and calculated relative to a known high titre serum (GL+) given an arbitary value of 100%. The distribution of results from this survey are shown in figure 3:1. The results represented a normal distribution curve with a positive skew. Sera of possible therapeutic use could be selected on the basis of a relative absorbance of 200% or greater (approximately 5 times the population mean absorbance value). Only 20 of the 695 donors (2.85%) had an absorbance greater than 200%. The majority of donors showed an absorbance of 80% or less and the mean and mode absorbances were approximately 45% and 30% respectively.

3:1:2. ELISA Assay of Normal Human Sera against Individual LPS Antigens.

Selected donor sera were assayed in a single antigen system against 31 purified lipopolysaccharides and lipid As. Nine S-LPS, 20 R-LPS,

FIGURE 3:1. Distribution of Anti-CGL IgG Antibodies in the Blood Donor Population.



and 2 lipid A were used in the screen as detailed in figure 3:2. Three sub-populations of sera were assayed: 10 high (>200%) in CGL-pool; 8 median (40%) in CGL-pool; and 8 low (less than or equal to 10%) in CGL-pool. Results for the 31 antigens and the CGL-pool are expressed in the form of a scatter diagram (figure 3:2) where absorbance values are representative of IgG levels. Much variation in levels of anti-LPS IgG in serum was seen within each sub-population (high, medium and low). In general, increasing mean absorbance to all antigens was seen with increasing CGL-pool absorbance. In addition, the range of absorbances showed overlapping values, but a stepwise increase was obtained against antigens alongside increasing anti-CGL values. Four rough LPS preparations showed only small differences in absorbances between the three sub-populations (*S. minnesota* Ra and Rb; *E. coli* R4; and *K. aerogenes* M10B). Other antigens (*S. typhimurium* Ra, Rb; *S. minnesota* O, Rd, Re; *E. coli* J5, K12 lipid A, O18:K-, O111, O86) showed higher absorbance range and mean in the sub-population with lowest CGL-pool values, than the median sub-population, but the high CGL-pool group remained highest.

3:1:3. Persistence of Anti-LPS IgG Levels in Blood Donor Sera.

Levels of IgG in monthly donations from selected plasmapheresis donors were measured against the previously-mentioned 31 LPS antigens in ELISA. As can be seen in figures 3:3a to 3:3h, levels of IgG in the CGL-pool assay and in individual antigen assays remained fairly stable over the period, and very few large changes in IgG levels (up or down) were observed. After these alterations IgG levels stabilised. By comparison of individuals, it can be seen that

FIGURE 3:2. Key to LPS Antigens.

No.	Code	Antigen
1.	St O	*S. typhimurium* wild type
2.	St Ra	" " " R1135
3.	St Rb	" " " R119
4.	St Rc	" " " R878
5.	St Rd	" " " R1032
6.	St Re	" " " R1102
7.	Mix-cocktail	CGL-pool
8.	Sm O	*S. minnesota* wild type
9.	Sm Ra	" " " R60
10.	Sm Rb	" " " R345
11.	Sm Rc	" " " R5
12.	Sm Rd	" " " R7
13.	Sm Re	" " " R595
14.	Sm lipid A	" " " lipid A
15.	Ec C62	*E. coli* C62
16.	Ec J5	" " " J5
17.	Ec K12	" " " K12
18.	Ec K12 Re	" " " K12 Re mutant
19.	Ec K12 lipid A	" " " K12 lipid A
20.	Ec R1	" " " R1
21.	Ec R2	" " " R2
22.	Ec R3	" " " R3
23.	Ec R4	" " " R4
24.	Ka M10B	*K. aerogenes* M10B
25.	PaC 605	*P. aeruginos* PAC605
26.	Ec O18K+	*E.* coli O18:K1
27.	Ec O18K-	" " " O18:K^-
28.	Ec O111:B4	" " " O111:B4
29.	Ec O6	" " " O6:K5
30.	Ec O16	" " " O16:K1
31.	Ec O86	" " " O86:K61
32.	Pa HABS-1	*P. aeruginosa* Habs type 1

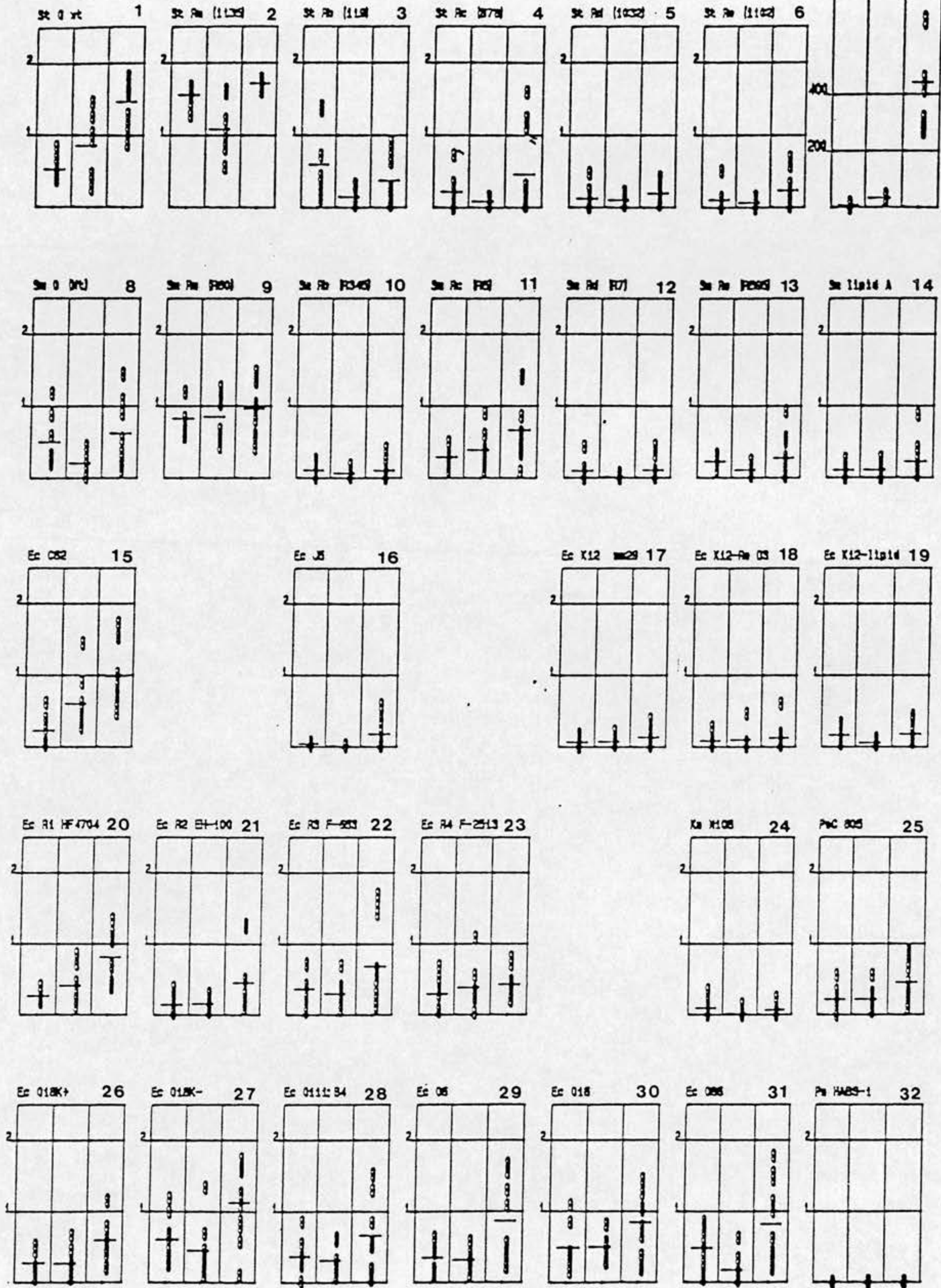


FIGURE 3:2. Reactivity in ELISA of three blood donor sub-populations with low, medium, and high levels of anti-CGL IgG antibodies (see antigen 7) against thirty one LPS antigens.

FIGURE 3:3.
Key to antigens in ELISA screen.

Organism	ELISA antigen
S. typhimurium wild type	St O wt
" " " R1542	St Ra
" " " R119	St Rb
" " " R878	St Rc
" " " R1032	St Rd
" " " R1102	St Re
S. minnesota wild type	Sm O (Wt)
" " " R60	Sm Ra (R60)
" " " R345	Sm Rb (R345)
" " " R5	Sm Rc (R5)
" " " R7	Sm Rd (R7)
" " " R595	Sm Re (R595)
" " " lipid A	Sm lipid A
E. coli R1	Ec R1
" " " R2	Ec R2
" " " R3	Ec R3
" " " R4	Ec R4
" " " J5	Ec J5
" " " K12	Ec K12 Dm34
" " " K12 Re	Ec K12 Re
" " " K12 lipid A	Ec K12 lipid A
" " " 06	Ec 06
" " " 016	Ec 016
" " " 018	Ec 018K-
" " " 018:K1	Ec 018K+
" " " 086	Ec 086
" " " 0111:B4	Ec 0111:B4
P. aeruginosa PAC605	PaC605
" " " Habs type1	Pa O-S1
K. aerogenes M10B	KaM10B
Mix-cocktail	CGL-pool

GGL-pool results presented as percentage value of control (1 = 100%, 2 = 200%). All other results presented as absorbance values at 590nm.

FIGURE 3:3a. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor COA-E.

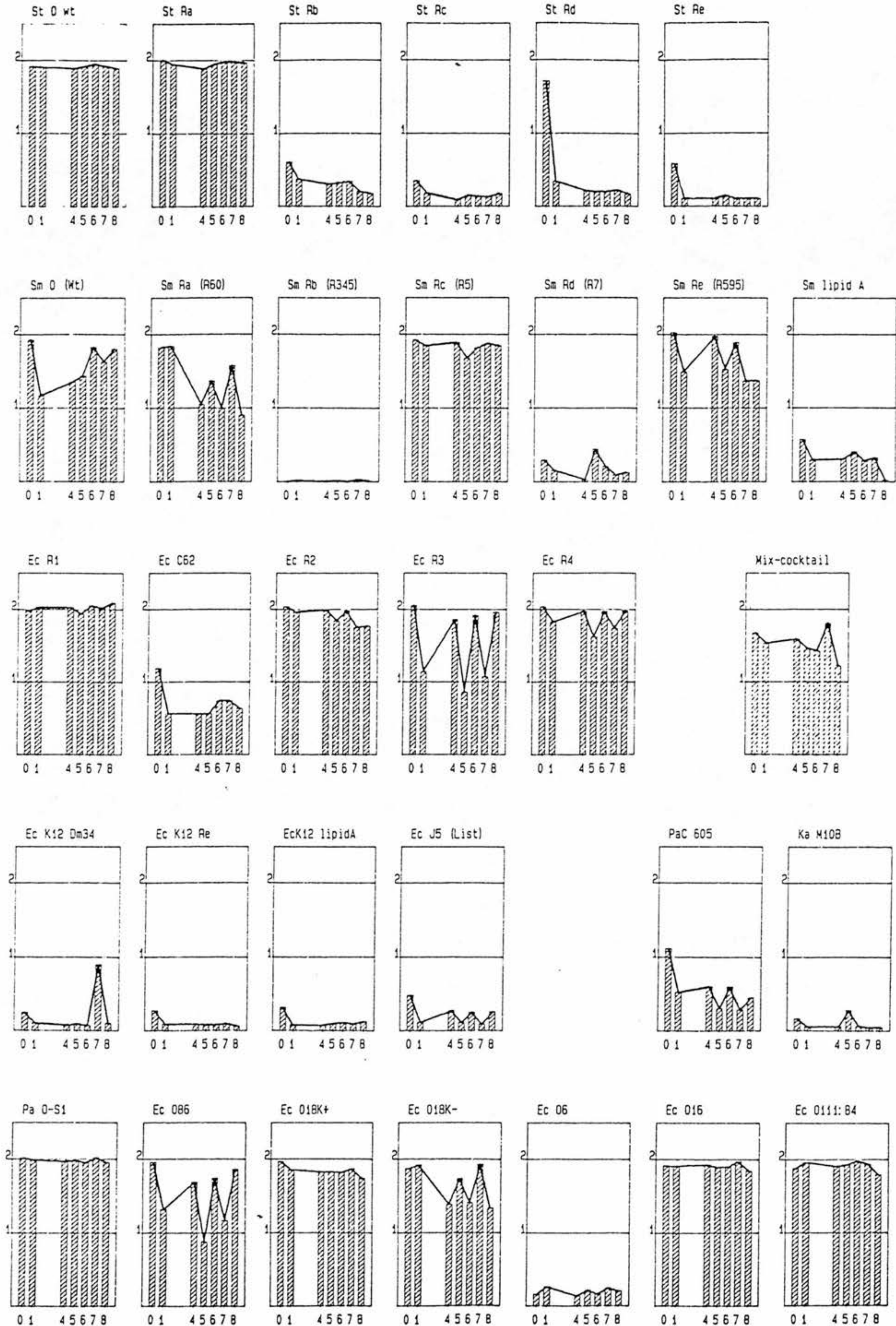


FIGURE 3:3b. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor GRO-D.

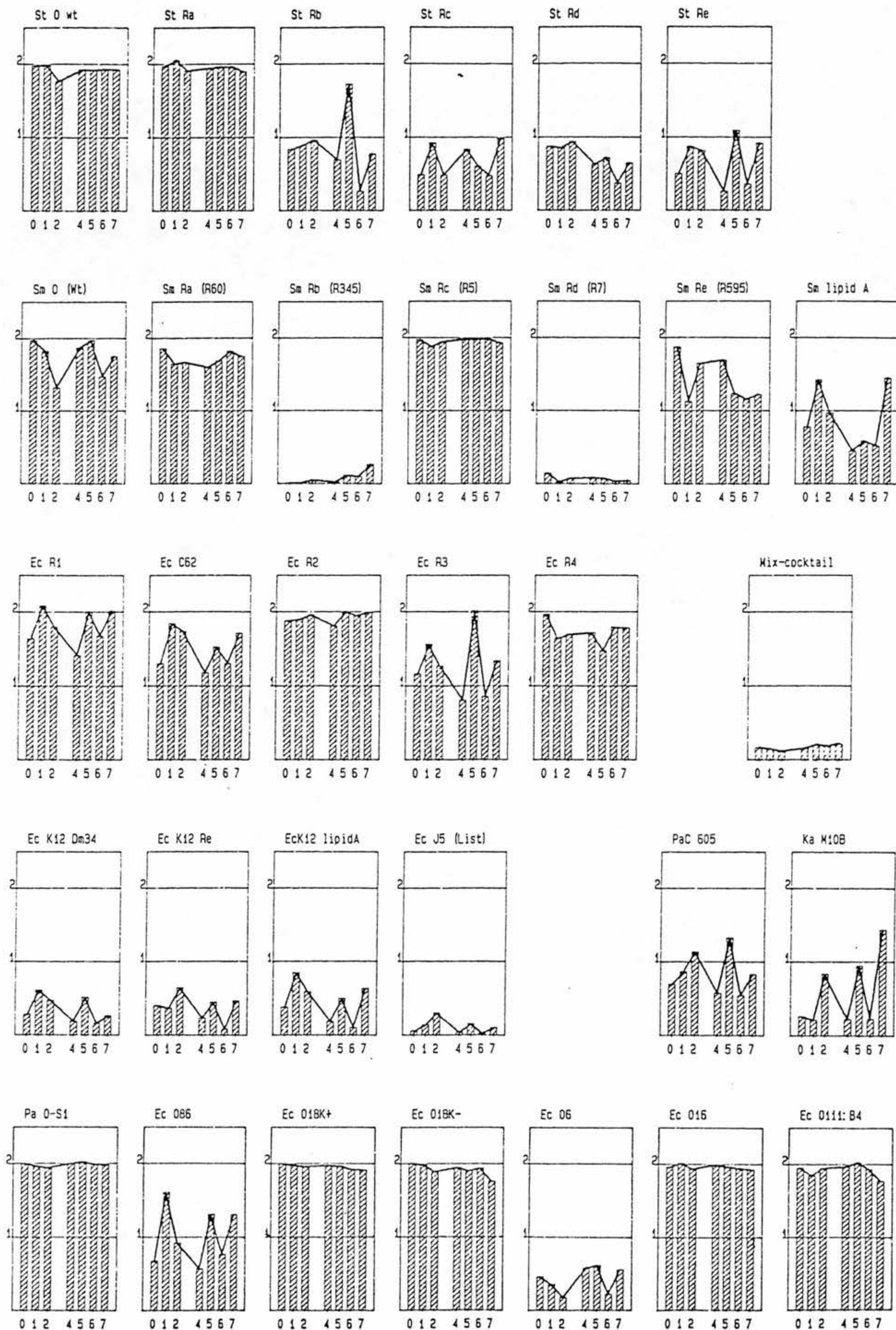


FIGURE 3:3c. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor CHA-N.

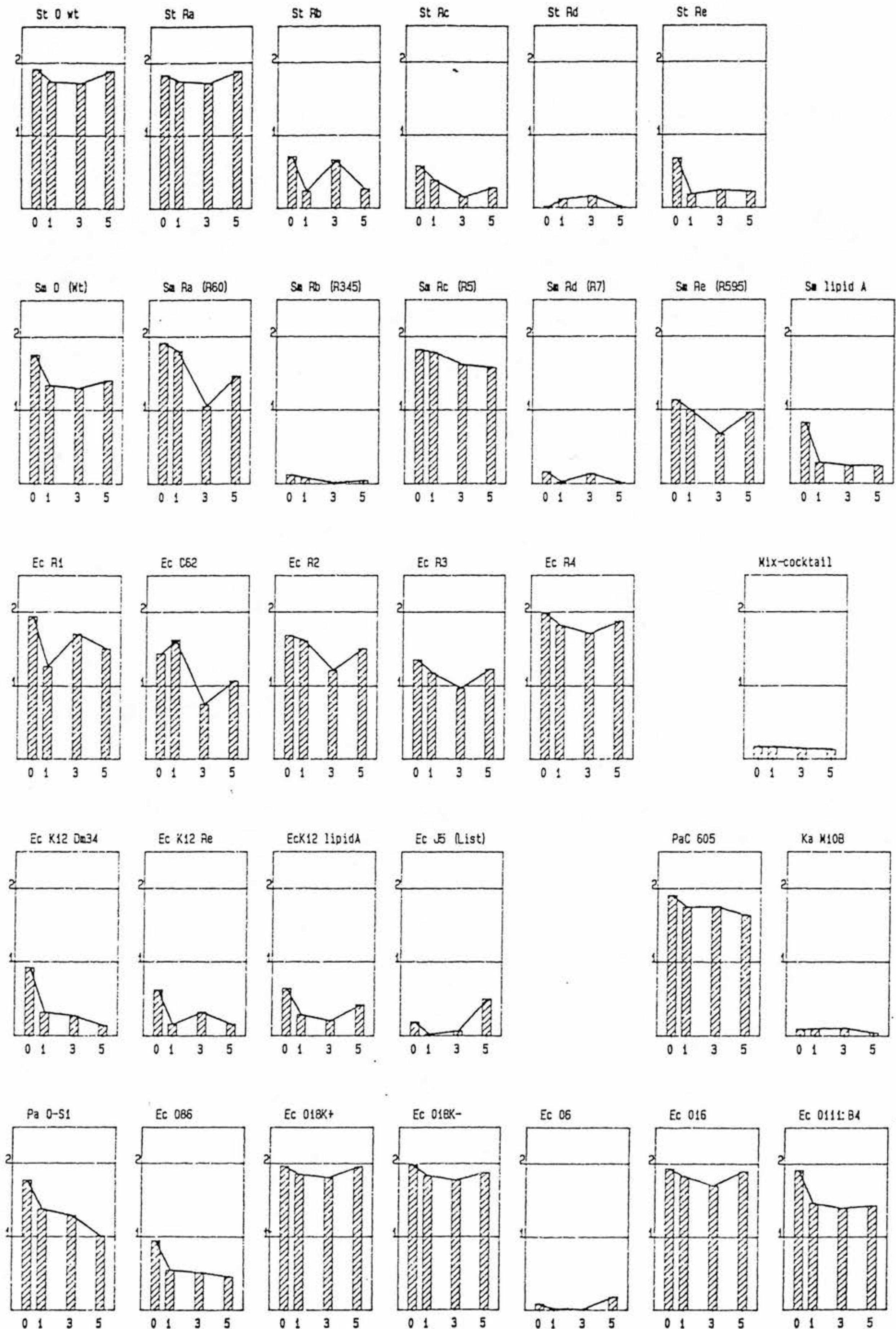


FIGURE 3:3d. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor LIP-C.

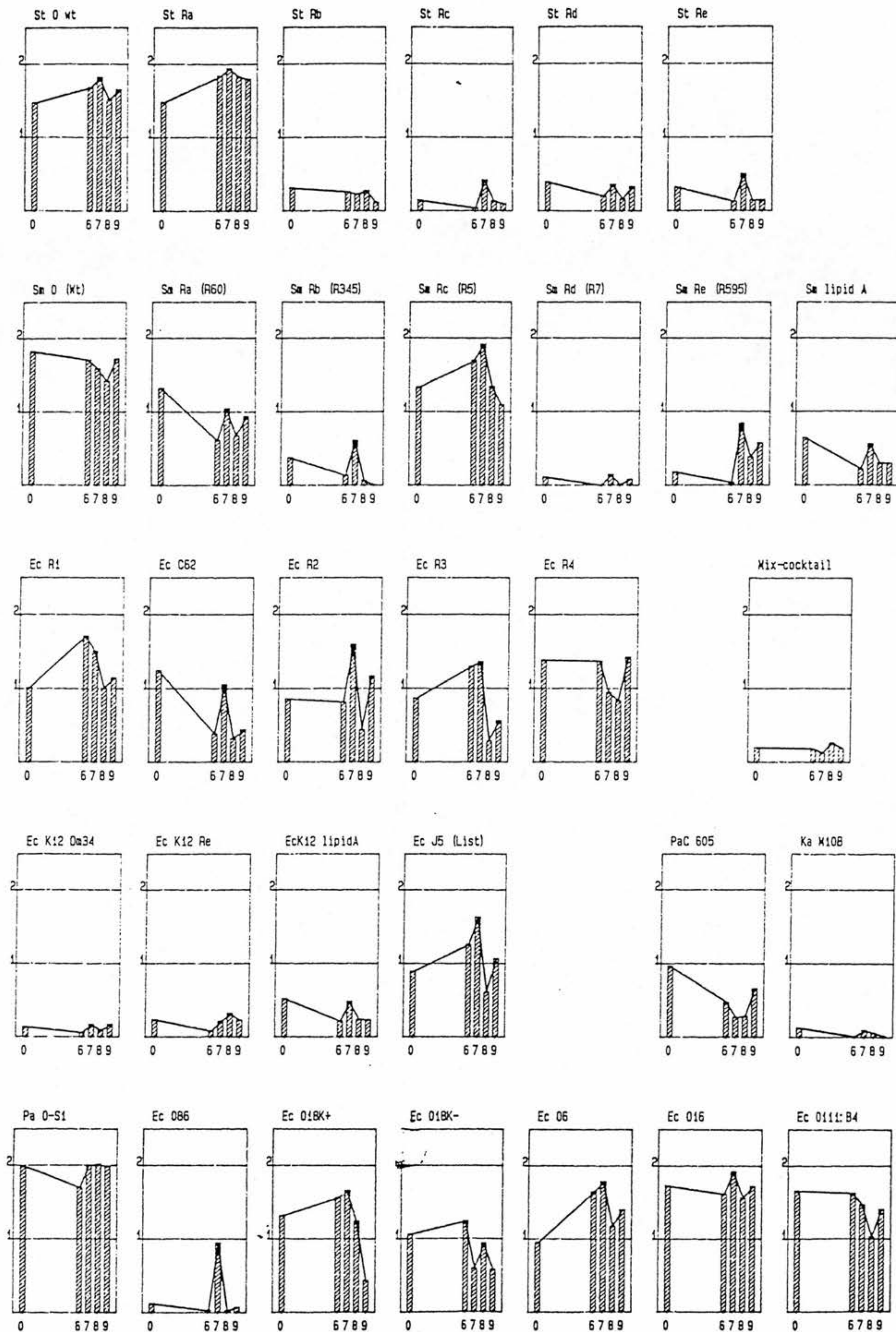


FIGURE 3:3e. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor GRA-D.

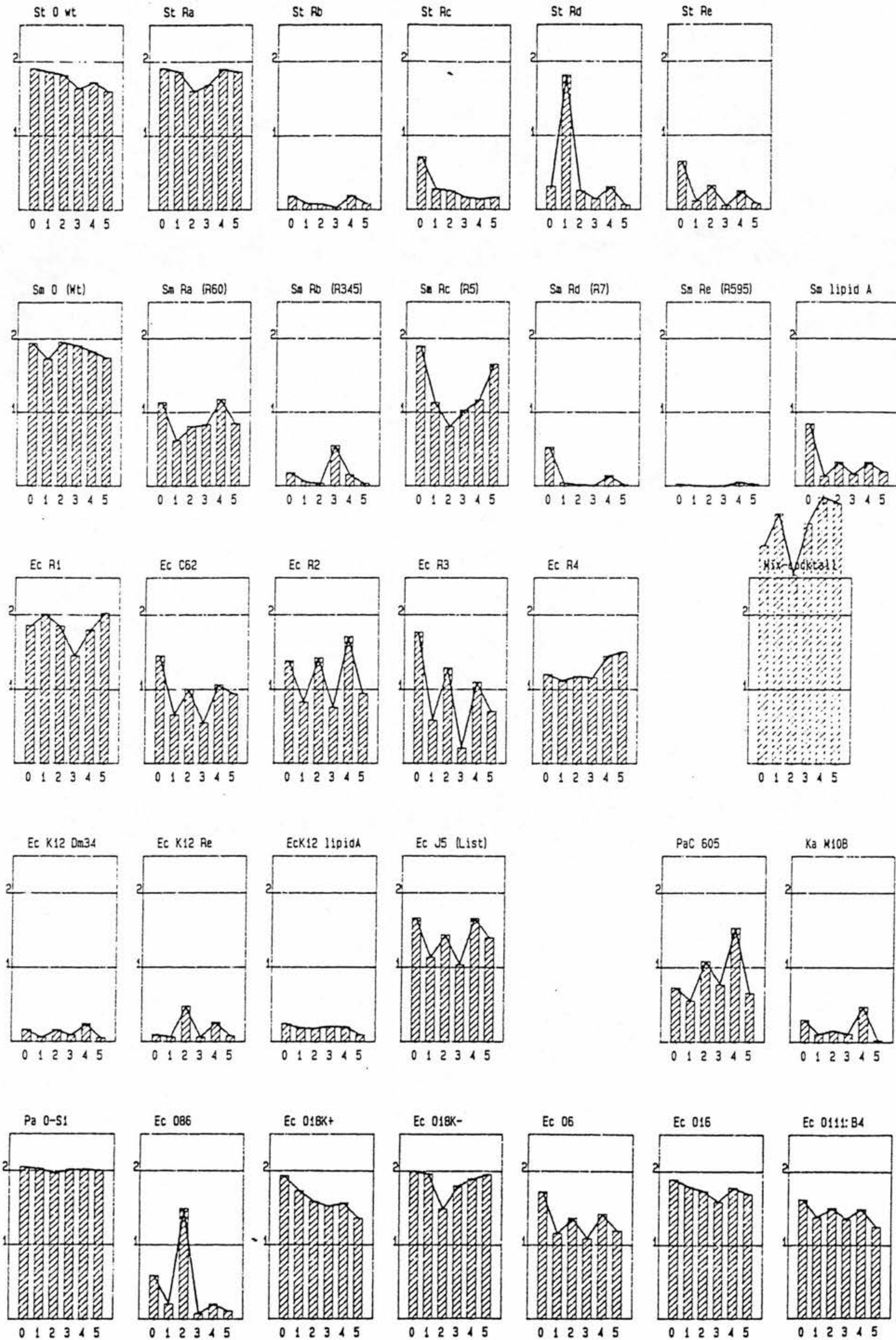


FIGURE 3:3f. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor PUR-A.

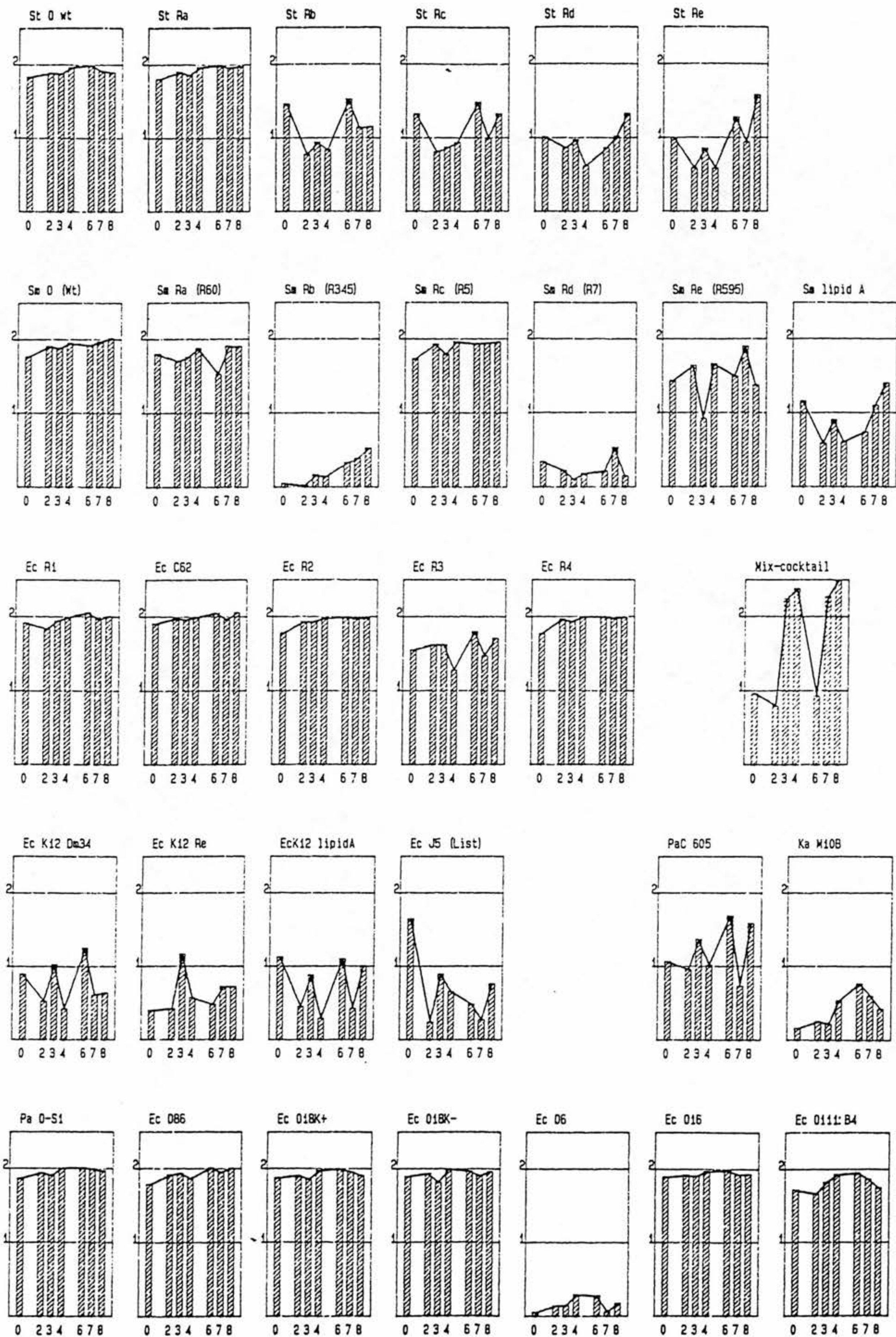


FIGURE 3:3g. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor PAP-J.

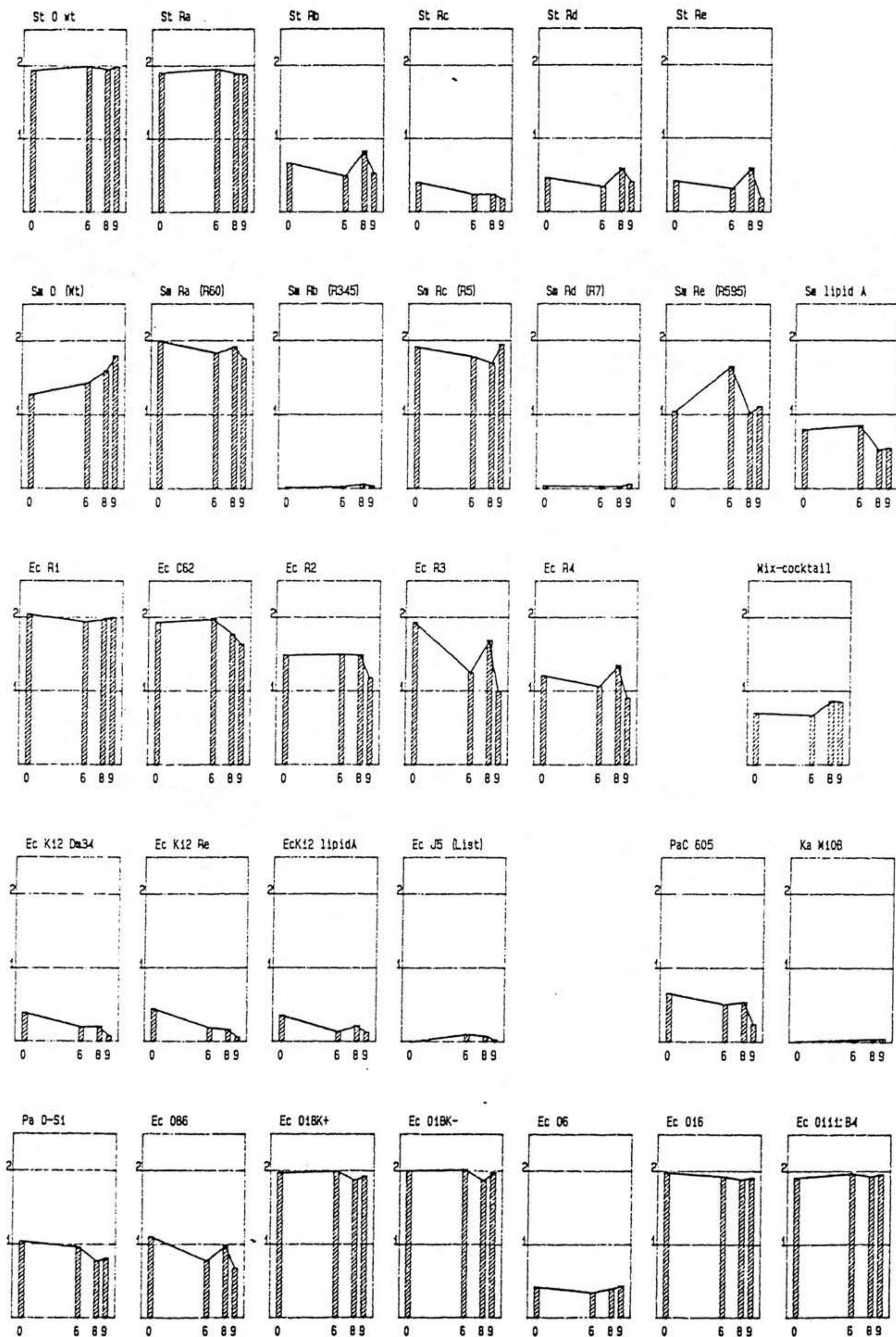
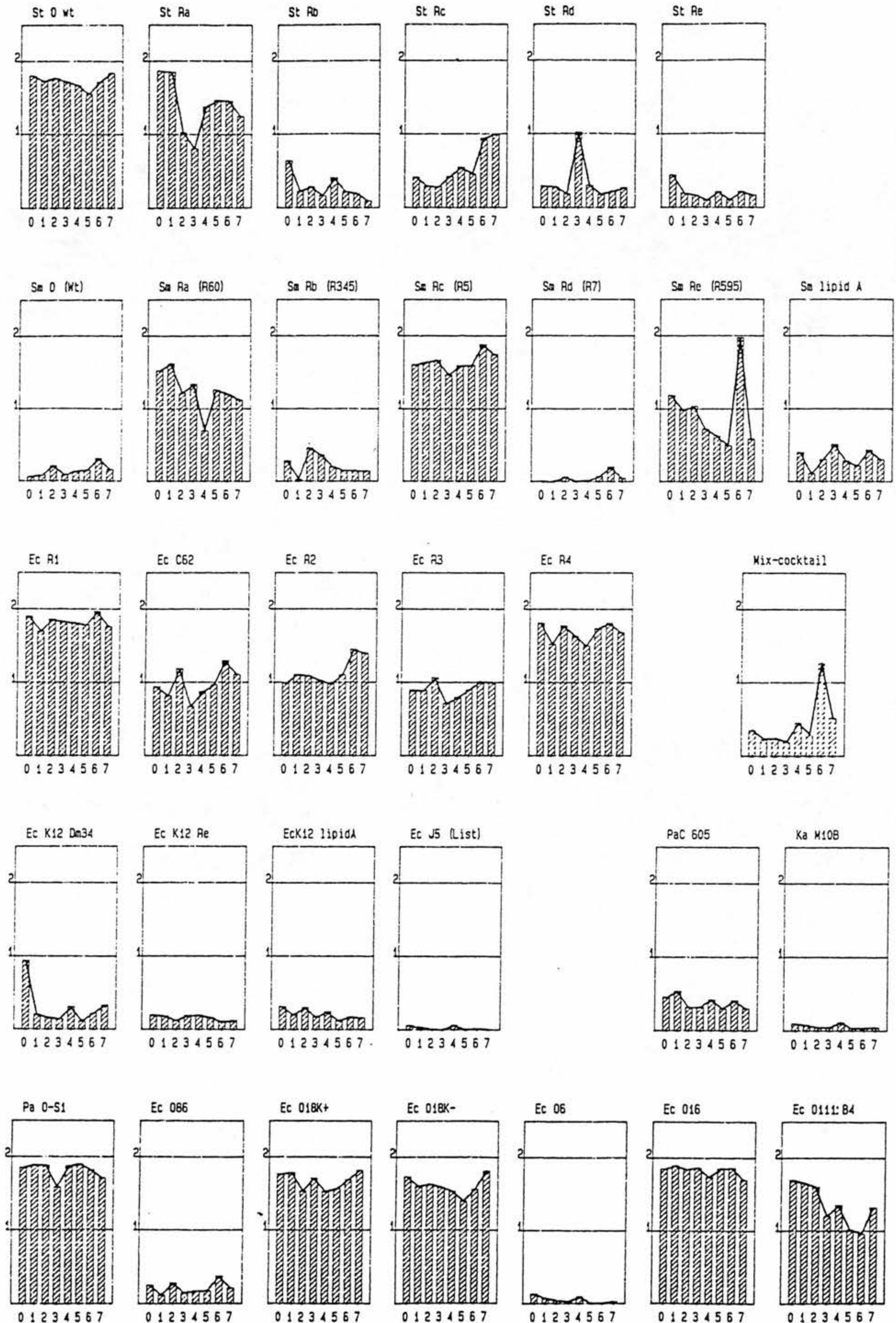


FIGURE 3:3h. Levels of Anti-lipopolysaccharide IgG in monthly serial samples from Plasmapheresis Donor AIT-N.



much variation existed in IgG levels to smooth and rough LPS, and to lipid A antigens, reflecting the variations observed in the CGL-pool assay. Antibody levels in individuals to both E. coli O18:K1 and E. coli O18:K$^-$ lipopolysaccharides showed little difference from each other at all points in all 8 donors. None of E. coli K12, K12Re, K12 lipid A, and K. aerogenes M10B showed major detectable levels of antibody in any of these 8 donors.

3:1:4. ELISA of IgG Prepared from Blood Donors.

Fractionation of 33 of the 695 sera was carried out to purify IgG components of sera as described in MATERIALS AND METHODS. After fractionation, the IgG preparations were subjected to ELISA to determine levels of antibody against the above mentioned series of LPS antigens. Histograms of the IgG profiles of 5 of these purified fractions are presented in figures 3:4a to 3:4e, and histograms for all 33 IgG fractions are present in appendix 1. Much variation in IgG levels existed between individuals and between different antigens within an individual. Some individuals possess very high levels of IgG to most antigens (e.g. numbers 5, 6 and 24), while others possess only low or undetectable levels of IgG (e.g. numbers 16, 26, and 33). In between these extremes a wide spectrum of levels of IgG was observed.

Of particular note was the different reactivities to Salmonella R-LPS molecules from S. minnesota and S. typhimurium. The binding of IgG to R-LPS of the same chemotypes did not produce comparable absorbance values for the two species.

Generally lower reactivity with LPS of Rb and Rd LPS chemotypes from S. typhimurium was observed in all of the IgG preparations than

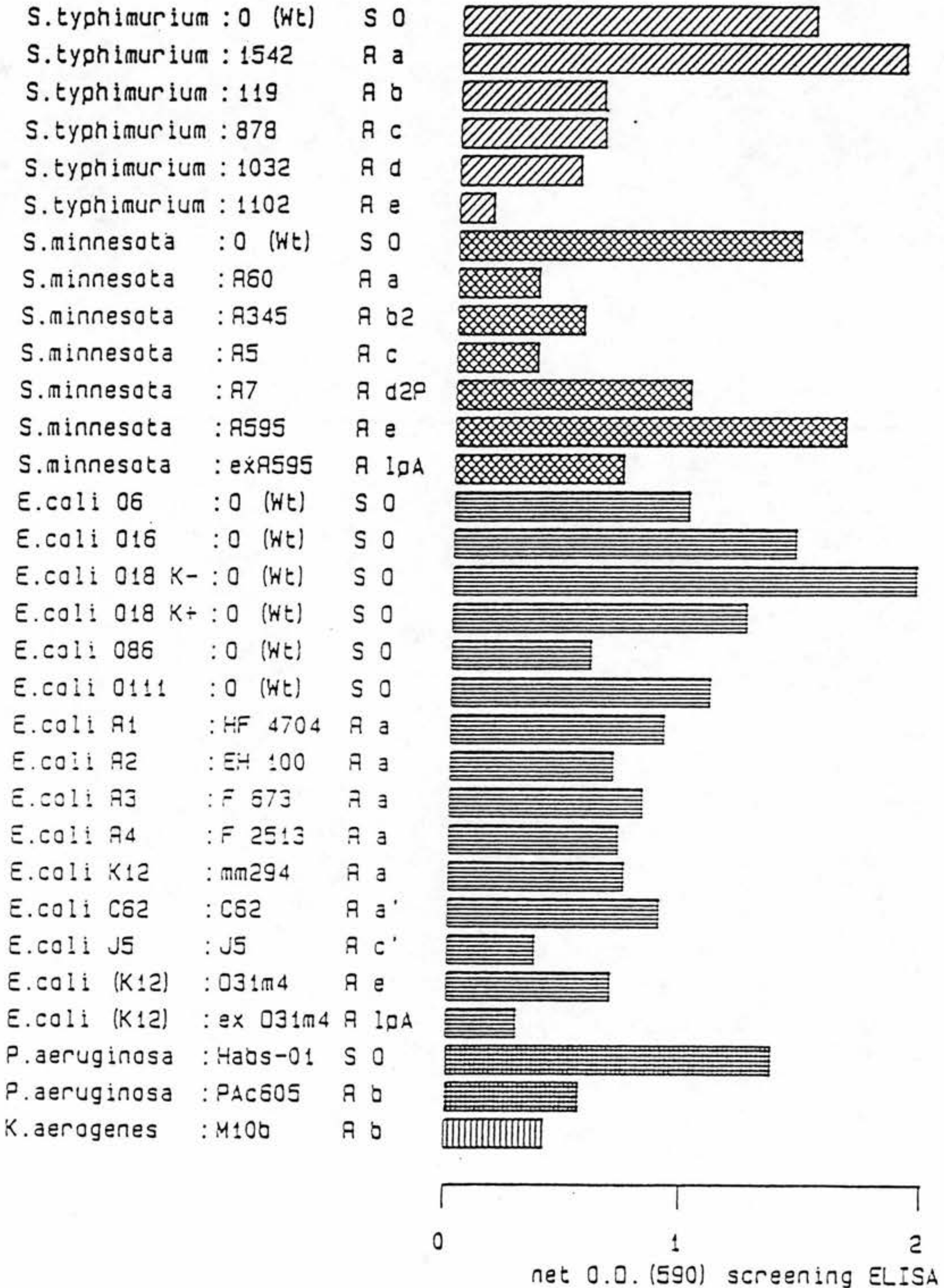


FIGURE 3:4a. Reactivity in ELISA of IgG (number 5) purified from human serum against 31 LPS antigens.

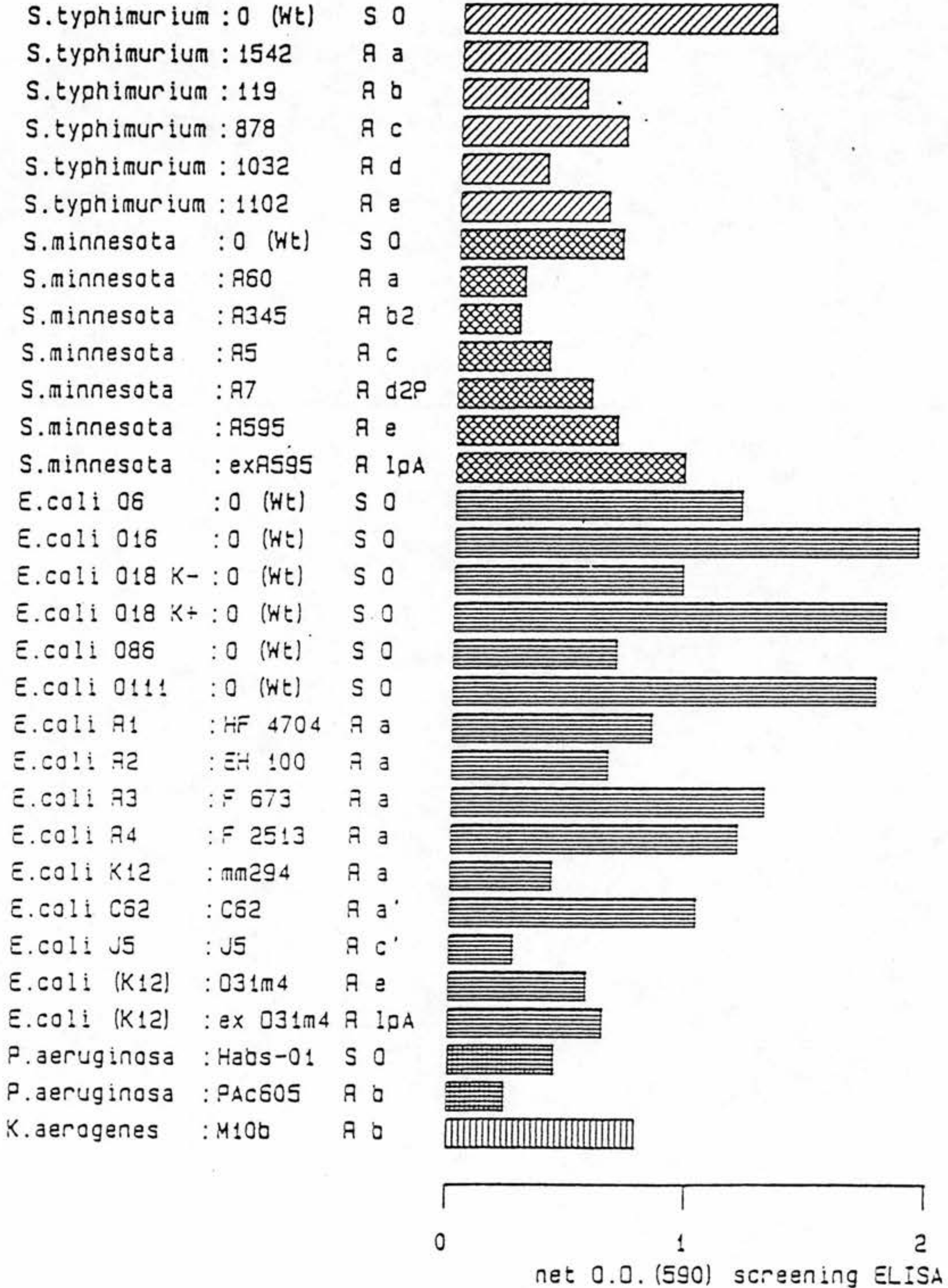


FIGURE 3:4b. Reactivity in ELISA of IgG (number 11) purified from human serum against 31 LPS antigens.

IgG-16

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Strain		
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: 031m4	R e
E.coli (K12)	: ex 031m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

FIGURE 3:4c. Reactivity in ELISA of IgG (number 16) purified from human serum against 31 LPS antigens.

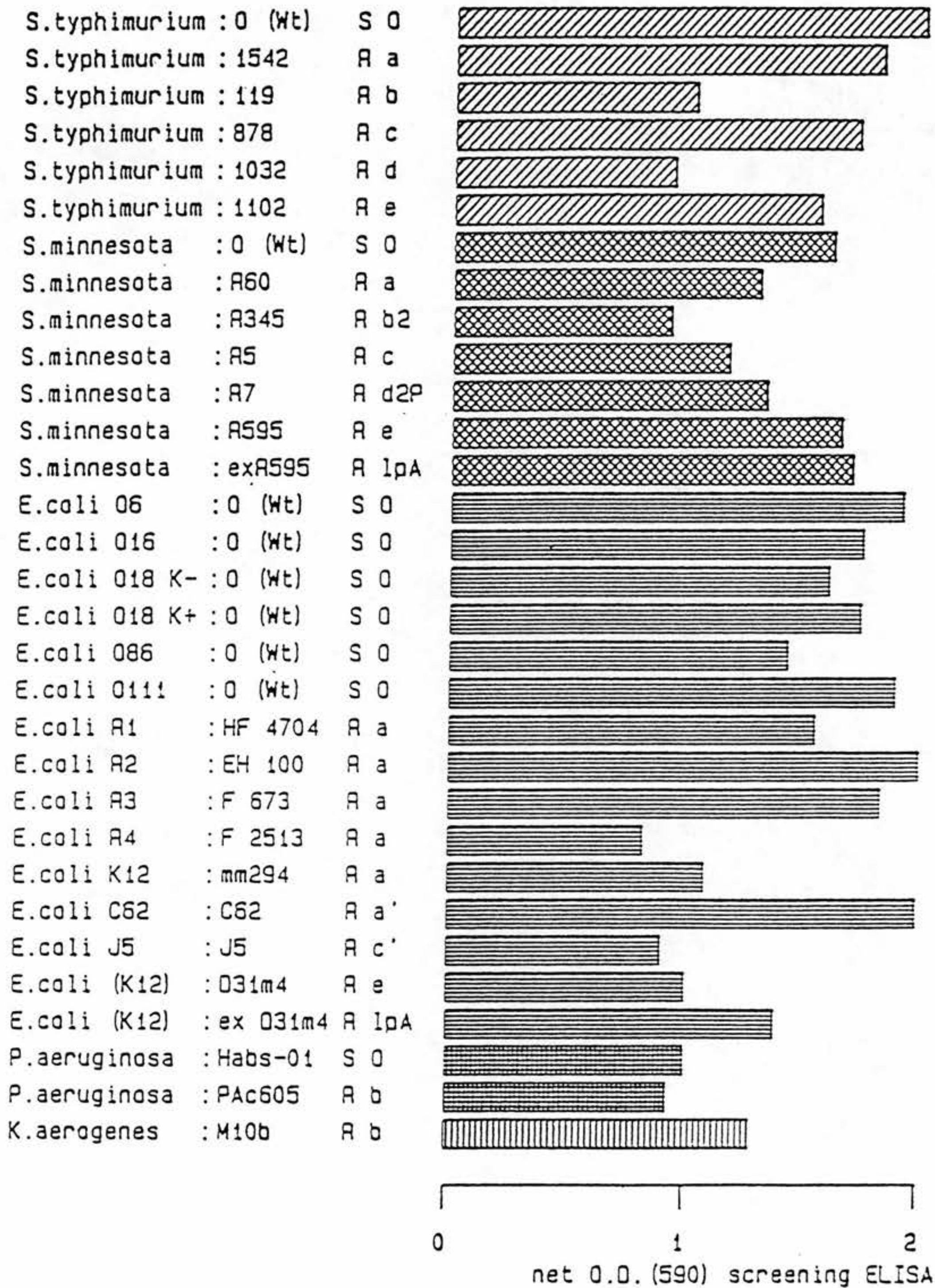


FIGURE 3:4d. Reactivity in ELISA of IgG (number 24) purified from human serum against 31 LPS antigens.

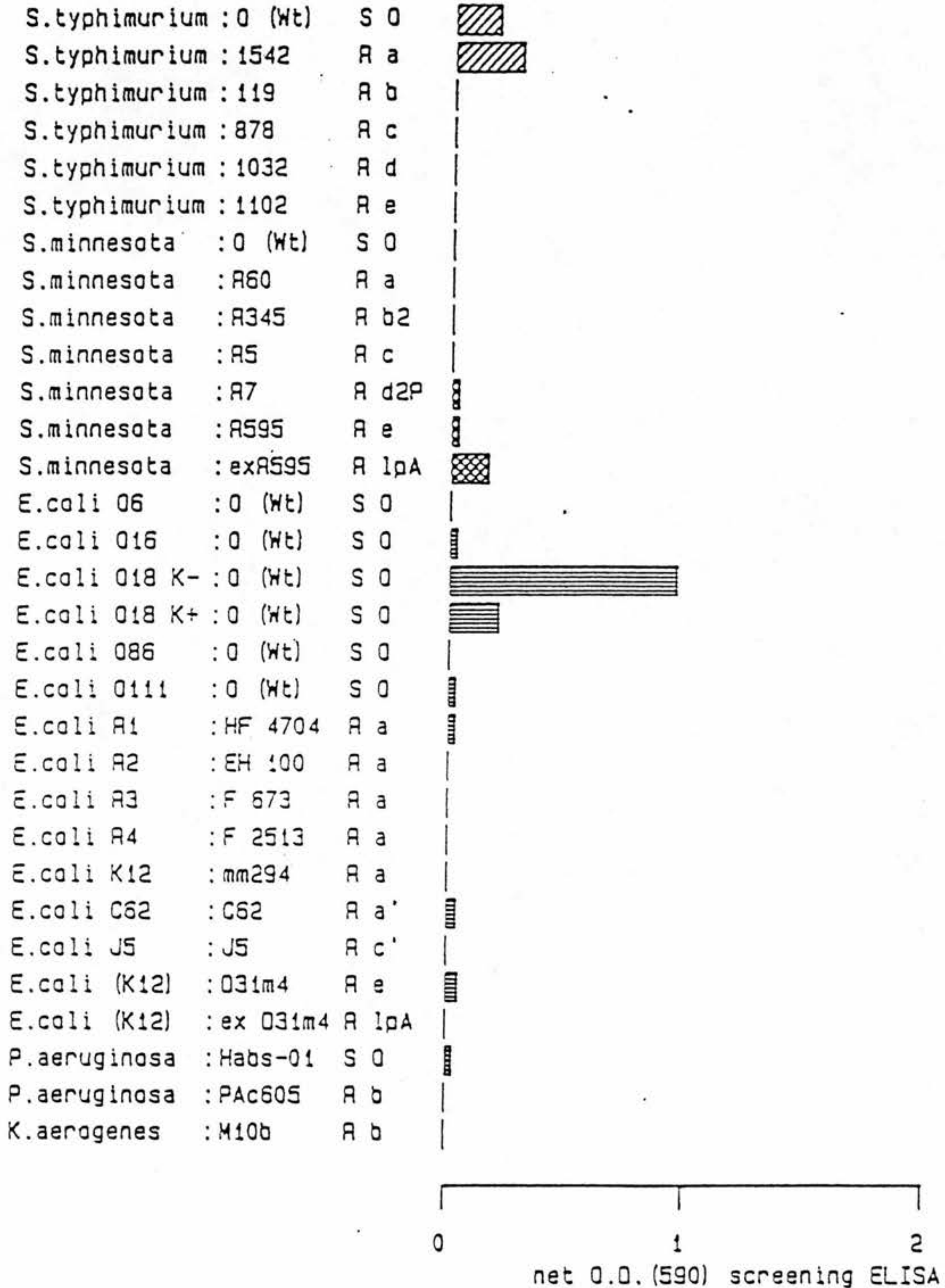


FIGURE 3:4e. Reactivity in ELISA of IgG (number 33) purified from human serum against 31 LPS antigens.

against Ra, Rc and Re chemotypes of LPS. Approximately 50% of the IgG preparations showed reactivity with Re LPS and Rc LPS, and the remaining 50% showed far greater reactivity with Rc than with Re LPS. These two sub-populations of antibodies were shown to be mutually exclusive in an assay of the 695 sera as detailed by Barclay and Scott (1987).

3:1:5. Immunoblotting of Immunoglobulins against LPS.

Lipopolysaccharides were separated on 14% SDS-free polyacrylamide gel as described in MATERIALS AND METHODS. LPS were initially visualised by silver staining PAGs (figure 3:5). Ladder patterns were clearly seen for smooth type LPS, and differentiation was obtained between core LPS of different chemotypes. Various immunoglobulins as described in MATERIALS AND METHODS were used to probe these antigens after electrophoretic transfer onto nitrocellulose.

a) Pseudomonas vaccinees IgG (PsV) was used at a dilution of 1:500 to probe lipopolysaccharides from P. aeruginosa Habs type 1 (S-LPS), P. aeruginosa PAC 605 (Rc-LPS), E. coli 086 (S-LPS), E. coli J5 (Rc-LPS), K. aerogenes M10B (Rb-LPS), S. typhimurium R878 (Rc-LPS), and S. typhimurium R1102 (Re-LPS) on nitrocellulose. The results are presented in figure 3:6a. IgG from this preparation bound strongly to LPS from P. aeruginosa Habs type 1 and S. typhimurium R878. Lesser reactivity was obtained against LPS from E. coli J5, K. aerogenes M10B, and high molecular weight components of E. coli 086 LPS. No reactivity was seen with P. aeruginosa PAC 605 or S. typhimurium R1102 lipopolysaccharides. This immunoblot was repeated against S. typhimurium R1542 (Ra), R119 (Rb), and R1032 (Rd) in

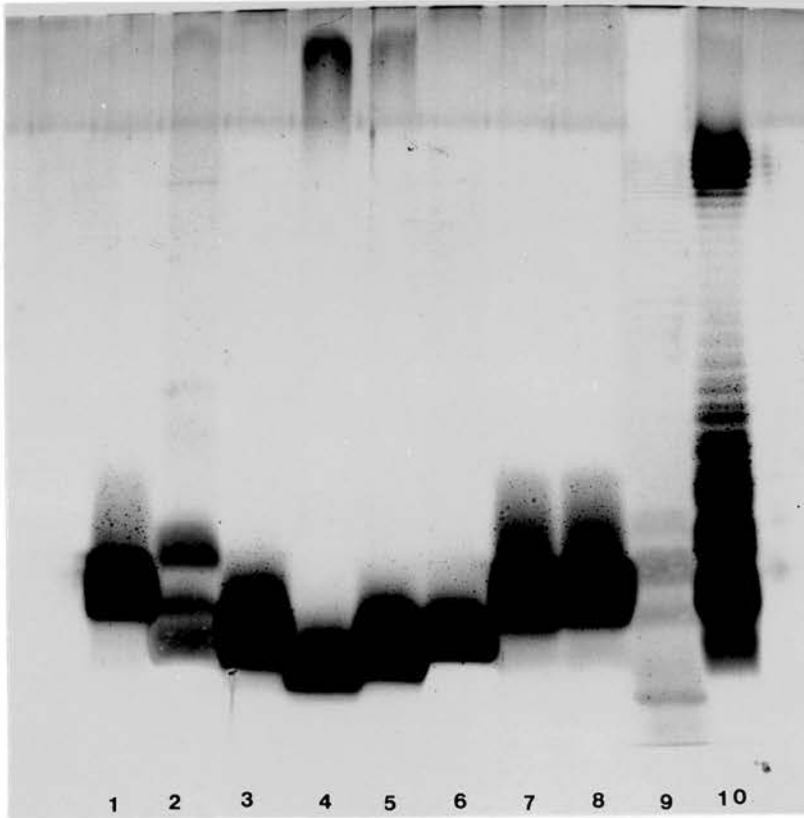


FIGURE 3:5. Silver stain of polyacrylamide gel (containing 14% acrylamide and no SDS) for lipopolysacchrides (10ug) from K. aerogenes M10B (track 1), P. aeruginosa PAC 605 (track 2), E. coli J5 (track 3), S. typhimurium R1102 - Re (track 4), S. typhimurium R1032 - Rd (track 5), S. typhimurium R878 - Rc (track 6), S. typhimurium R119 - Rb (track 7), S. typhimurium R1542 - Ra (track 8), P. aeruginosa Habs type 1 (track 9) and E. coli 086:K61 (track 10).

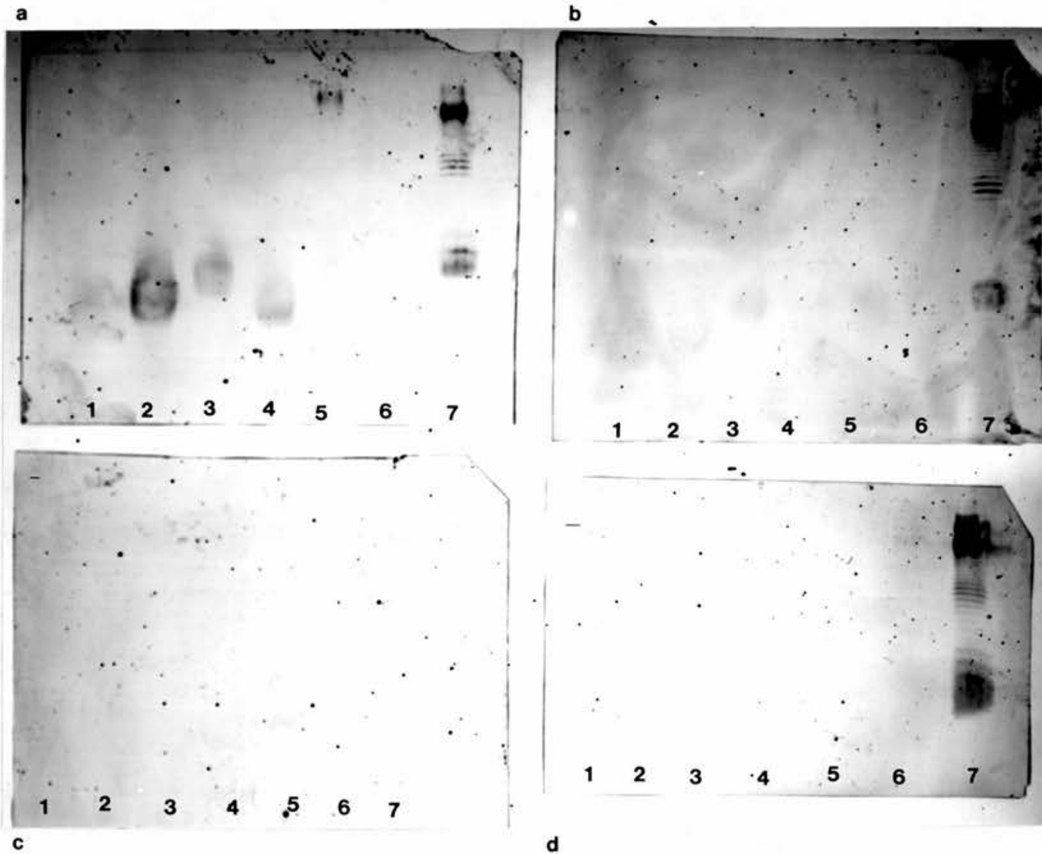


FIGURE 3:6a-d. Immunoblot of Pseudomonas vaccinees IgG (PsV - figure a), Pseudomonas positive IgG (Ps+ - figure b), Pseudomonas negative IgG (Ps- - figure c), and a high titre human donor serum (RAC+ - figure d) at dilutions of 1:500 against lipopolysaccharides transferred from a 14% SDS-free polycrylamide gel onto nitrocellulose. Lipopolysaccharides from S. typhimurium R1102 - Re (track 1), S. typhimurium R878 - Rc (track 2), K. aerogenes M10B (track 3), E. coli J5 (track 4), E. coli 086:K61 (track 5), P. aeruginosa PAC605 (track 6), and P. aeruginosa Habs type 1 (track 7) were used.

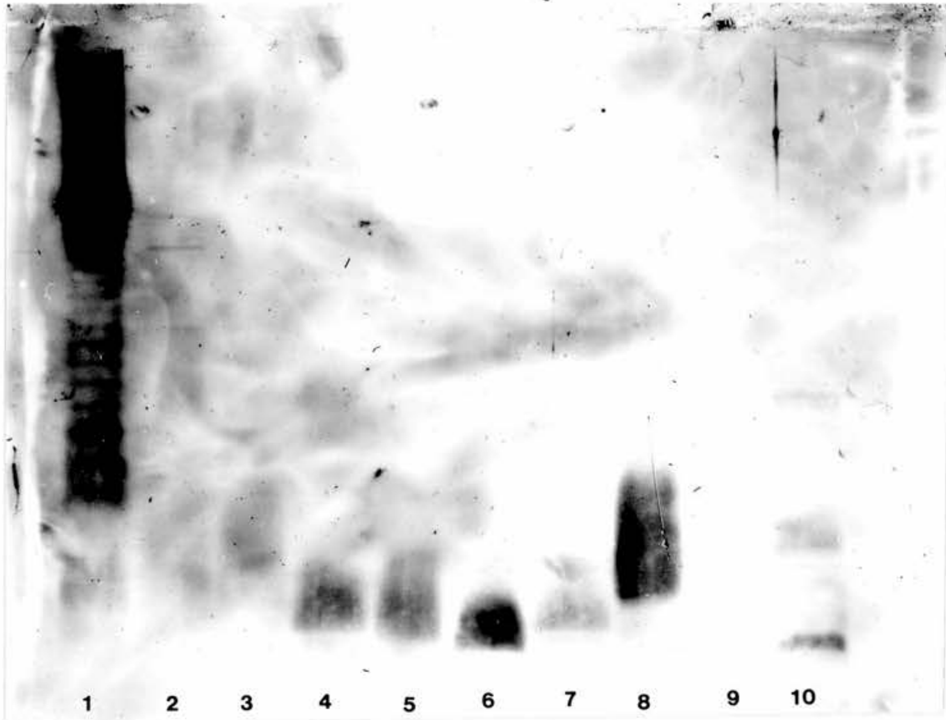


FIGURE 3:6e. Immunoblot of antigens transferred onto nitrocellulose from a 14% SDS-free polyacrylamide gel with Pseudomonas vaccinees IgG (PsV) at a dilution of 1:500. The antigens were lipopolysaccharides (10ug) purified from E. coli 086:K61 (track 1), S. typhimurium R1542 - Ra (track 2), S. typhimurium R119 - Rb (track 3), S. typhimurium R878 - Rc (track 4), S. typhimurium R1032 - Rd (track 5), S. typhimurium R1102 - Re (track 6), E. coli J5 (track 7), K. aerogenes M10B (track 8), P. aeruginosa Habs type 1 (track 9), and P. aeruginosa PAC605 (track 10).

addition to the above antigens. The results (figure 3:6e) show that PsV once again exhibited wide cross-reactivity.

b) Pseudomonas positive IgG was diluted 1:500 and was reacted as in (a) against the initial panel of seven LPSs. In this case, only antibodies directed towards the O-antigen of P. aeruginosa Habs type 1 were detectable (figure 3:6b).

c) Pseudomonas negative IgG was used as above, and as shown in figure 3:6c, no binding to any of the 7 LPS antigens was detectable.

d) A high titre human donor serum (RAC+) was assayed against the antigens used in (a), (b), and (c) above. Figure 3:6d shows that this serum contained IgG which bind to LPS from P. aeruginosa Habs type 1, but to no other LPS antigen in this assay.

e) Further immunoblots were carried out against LPS from E. coli O86 and J5, P. aeruginosa Habs type 1 and PAC605, and S. typhimurium R1542, R878, R1102, and lipid A (prepared by hydrolysis of R878 LPS for 1h in 1% v/v acetic acid) with sera which were assayed in the CGL-pool ELISA as either high or low, with the inclusion of antigen dot (2ul) controls. Little activity to any R-LPS was obtained in either high or low titre sera. Major binding was observed to LPS from P. aeruginosa Habs type 1 from sera of both high and low titre which were examined (figures 3:7a and 3:7b). Additionally, binding to none of the antigens was obtained in certain high and low titre sera.

f) An avidin-biotin system and an alkaline phosphatase system were also used in an attempt to increase sensitivity of results, but no increase was obtained.

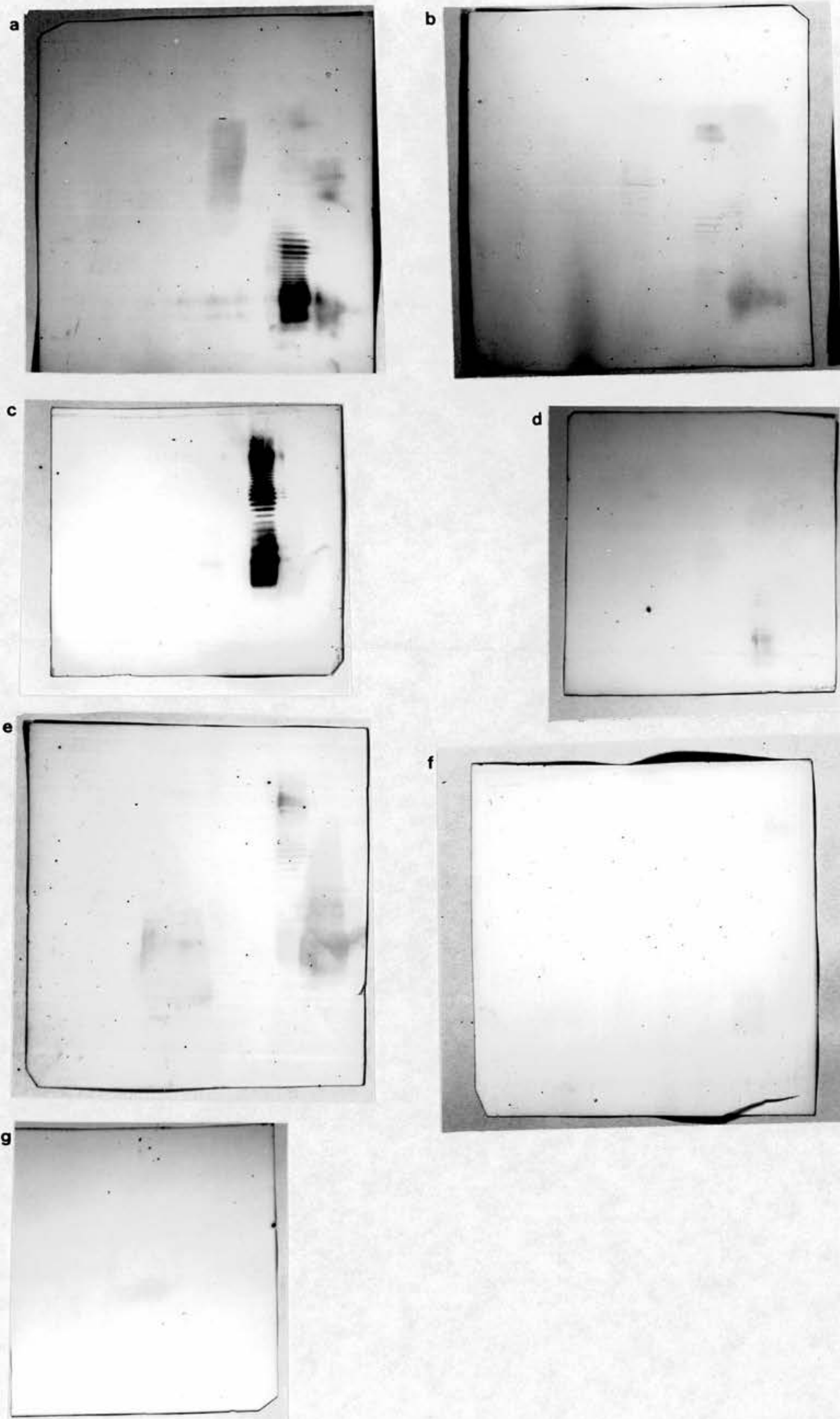


FIGURE 3:7. Immunoblot of normal human sera selected as positive (figures a-d) and negative (figures e-g) in ELISA against lipopolysaccharides (10ug) from S. typhimurium R1102 - Re (track 1), P. aeruginosa PAC605 (track 2), S. typhimuriium R878 - Rc (track 3), E. coli J5 (track 4), K. aerogenes M10B (track 5), S. typhimurium R1542 - Ra (track 6), P. aeruginosa Habs type 1 (track 7), and E. coli O86:K61 (track 8).

3:2. Endotoxin and Immunoglobulin Assays of Human Shock Patient Sera.

Six patients were available for study, but from three less than 10 samples of serum were obtained and will not be considered. Endotoxin concentrations were calculated by use of a Limulus amoebocyte lysate (LAL) assay, and IgG levels to 31 lipopolysaccharide and lipid A antigens were assayed in a polymyxin-ELISA system as described in MATERIALS AND METHODS. Results are presented as graphs (figures 3:8 to 3:10) of both endotoxin and antibody levels to 12 of the 31 antigens plotted against time (the results for the remaining antigens are presented in appendix 2). Endotoxin levels are expressed in endotoxin units per millilitre (EU/ml) and IgG levels as absorbance at 590nm. Also represented in the graphs are the points at which patients received infusions of blood products (fresh human plasma or packed red blood cells).

i) Patient BS: Thirty-two samples were obtained over a period of 34 days (figures 3:8a to 3:8d). Low levels of LAL activity were detectable initially (approx. 2.0EU/ml), which fell over a period of 5 days to levels at or below the detection limit of the assay (0.5 EU/ml, equivalent to 6pg/ml). After day 11, a rise in LAL activity was obtained, with a peak of 6.9EU/ml (82.8pg/ml) on day 14. Levels of endotoxin remained detectable at approximately 1.5EU/ml from days 16 to 23 with a small peak of activity (2.0 EU/ml) on day 22. After this point, endotoxin levels remained at the lower limit of detection except for a further small peak on day 32.

Anti-LPS IgG levels to all antigens examined showed considerable fluctuation from days 0 to 10, during which period the patient was receiving infusions of blood products. The first eleven days

FIGURES 3:8 to 3:10. Key to LPS Antigens.

A	*S. minnesota*	Rc	R5
B	" " "	Re	R595
C	" " "	lipid A (from R595)	
D	" " "	smooth	
E	" " "	Ra	R60
F	*E. coli*	R1	
G	" " "	R2	
H	" " "	R3	
I	" " "	R4	
J	" " "	K12	
K	" " "	06	
L	" " "	016	
M	" " "	018	
N	" " "	086	
O	" " "	0111	

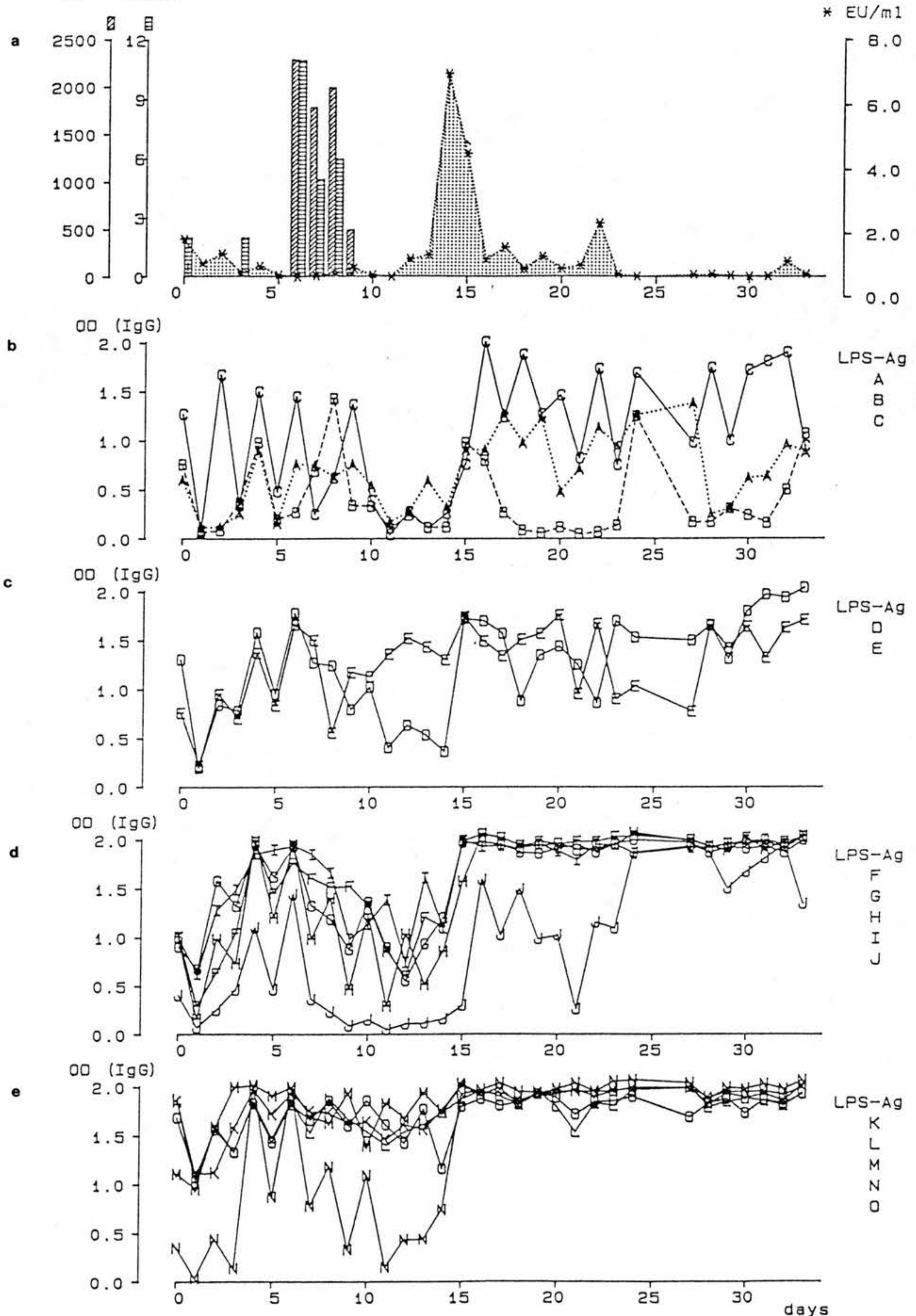


FIGURE 3:8. Activity in Limulus Amoebocyte Lysate Assay in serum from a Patient with Septic Shock (graph a) with relevant infusions of blood products, and Levels of IgG antibodies to 15 LPS antigens (graphs b-e). LPS antigns are described on the page opposite.

produced simultaneous rises and falls in IgG to rough lipopolysaccharides and to E. coli 086 LPS. Antibodies to E. coli O-serotypes 06, 016, 018 and 0111 remained fairly high and relatively stable from day 0 to day 10.

Between days 11 and 14 a general depression of IgG levels to rough LPS (with the exception of S. minnesota Ra) was observed, coinciding with the large peak of endotoxin activity. IgG levels to all E. coli smooth LPS increased over this period. Levels of anti-S. minnesota Re became noticeably depressed prior to each peak of LAL activity, and rose as levels of endotoxiin fell.

With the reduction in endotoxin from day 15, an increase was obtained in IgG to all rough LPS and to lipid A. The only exception to this was IgG to S. minnesota Re which remained depressed until day 24. IgG to Ra and to smooth LPS remained reasonably stable until the final sample, although a small drop in anti-Ra to moderate levels was obtained between days 21 and 27 with levels recovering by day 28, while IgG versus Re, Rc and lipid A showed larger fluctuations. A rising trend could, however, be seen after a second depression of IgG to Re and Rc on day 27.

ii) Patient MCC: Figures 3:9a-e indicate that this patient had high levels of LAL activity initially (12 EU/ml), falling to approximately 5 EU/ml on days 2 and 3, followed by a return to 12 EU/ml on day 5. After this point levels of endotoxin fell with a small peak of 4 EU/ml on day 9. Reduction of endotoxin levels corresponded approximately with infusion of packed red blood cells.

Levels of IgG to lipopolysaccharides were initially low to negligible, with the exception of E. coli R2 and R4 which were moderate. All antibodies showed a continuous rise to intermediate or

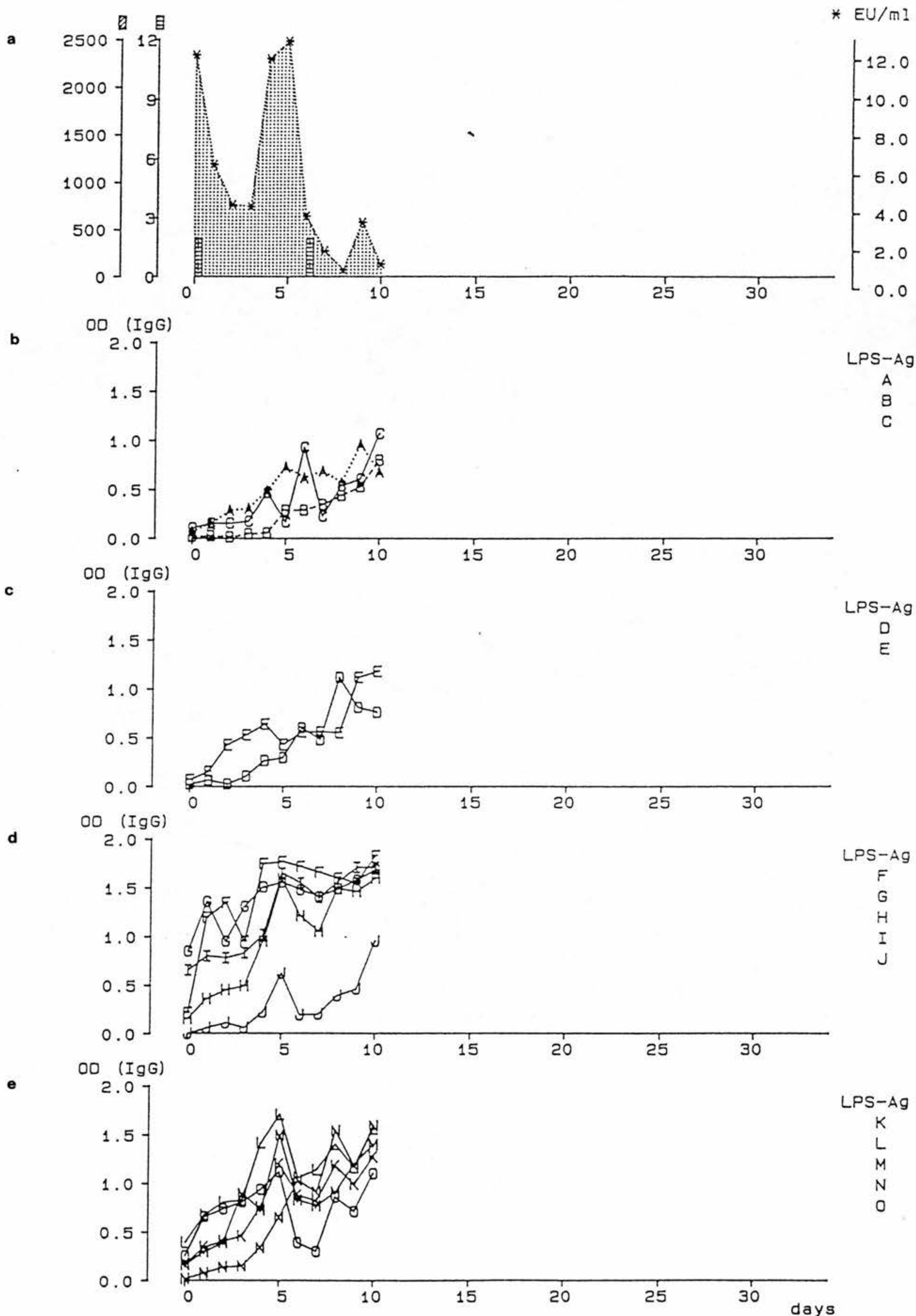


FIGURE 3:9. Activity in Limulus Amoebocyte Lysate Assay in serum from a Patient with Septic Shock (graph a) with relevant infusions ofblood products, and Levels of IgG antibodies to 15 LPS antigens (graphs b-e). LPS antigens are described opposite page 135.

high levels with IgG to E. coli R1, R2 and R4 reaching plateaux on day 5. Antibodies to E. coli LPS (rough and smooth) show steep rises during the second peak of endotoxin, followed by a drop as endotoxin levels fall. From this point onwards (day 7), antibodies climb again.

For the Salmonella minnesota mutants, the rise in IgG is slower, with anti-Re being the last to recover as the peak of endotoxin on day 5 falls.

iii) Patient MCM: No endotoxin was detectable until day 5 of the study (figure 3:10). Levels of endotoxin fluctuated after this point, reaching a low of 1.0 EU/ml on days 10 and 13, and rising to a peak on day 15. Levels remained elevated although a small fall was seen between days 15 and 21.

Antibodies to S. minnesota rough LPS were detectable only at low or negligible levels between days 0 and 6 with the exception of IgG to Rc LPS which was present at moderately high levels. A small peak also occurred for lipid A prepared from S. minnesota corresponding with the first detectable LAL result. IgG levels to E. coli rough LPS were far more variable but remained approximately constant. E. coli 06, 016 and 0111 IgG showed declining trends from moderate to low levels, and IgG binding to 086 became negligible. E. coli 018, on the other hand, showed IgG levels increasing from moderate to high levels by day 3, after when stabilisation occurred. Between days 6 and 9 all IgG increased to moderate or high levels concomitant with infusion of packed reb blood cells on day 6. Days 10 and 11 produced a depression of IgG to near baseline levels just prior to a large rise in LAL activity from day 14 onwards. After this point, antibodies recognising S. minnesota remained negligible,

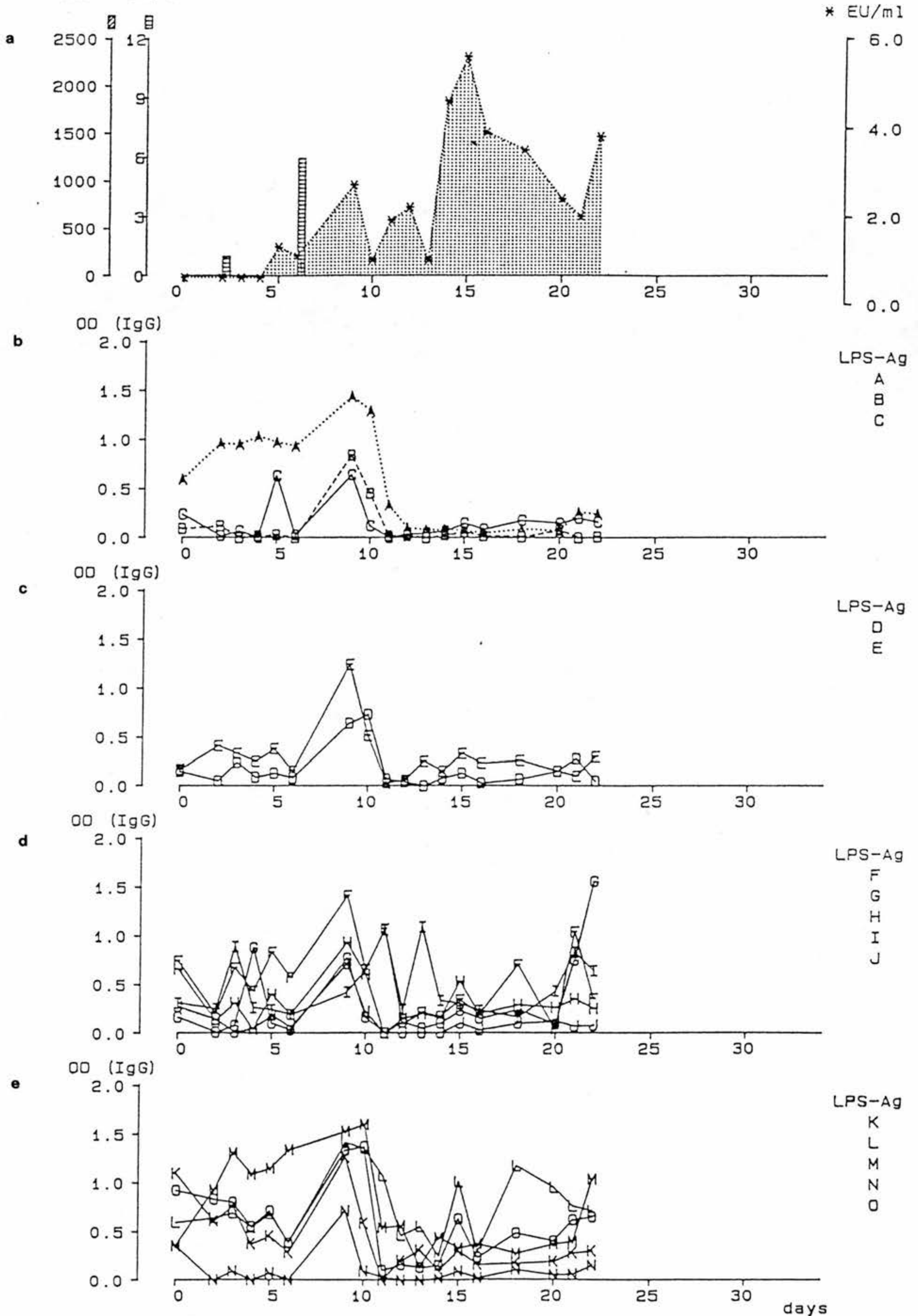


FIGURE 3:10. Activity in Limulus Amoebocyte Lysate Assay in serum from a Patient with Septic Shock (graph a) with relevant infusions of blood products, and Levels of IgG antibodies to 15 LPS antigens (graphs b-e). LPS antigens are described opposite page 135.

as did those against *E. coli* K12, R2, R3, 06, 018 and 086. IgG against *E. coli* R1 and R4 remained moderate but large fluctuations were visible. *E. coli* 016 recovered to moderate levels followed by a gradual decline after day 18, and after a temporary depression anti-0111 IgG increased by a small increment during the large peak of endotoxaemia. Rises to high and moderate levels were obtained in IgG to *E. coli* R2 and 018 respectively on days 21 and 22.

3:3. Longitudinal Study of Immunoglobulin Response to LPS of Rabbits Immunised with Bacteria.

Dutch rabbits were challenged intravenously at 28 day intervals with approximately 10^8 heat-killed bacteria suspended in 1.0ml of sterile PBS. The immunisation schedules were carried out as detailed in table 3:1. Lipid A bacteria were prepared by hydrolysis of cells in 1% acetic acid by heating for 90 min at 100°C, followed by three washes in PBS to remove acetic acid and hydrolysed components.

Serum samples were assayed in the ELISA system described in MATERIALS AND METHODS for anti-lipopolysaccharide IgG and IgM. Serum was diluted 1:100 in antibody buffer, and anti-rabbit IgG-urease was used for detection.

3:3:1. RABBIT 130.

This rabbit was immunised with a series of rough type cells from *S. typhimurium* in ascending LPS size from lipid A to Ra. Figure 3:11a-g indicate the alterations in levels of IgG.

Pre-immunisation levels of IgG to all 26 antigens examined were negligible. Immunisation with lipid A cells produced small rises against *S. minnesota* lipid A; *S. typhimurium* Ra and Rc; *E. coli* R1, R2, R4, K12Re, K12 lipid A, O-types 2, 6, 18, 75, 86, and 111; and *S. typhimurium* wild type. Most of these responses were boosted upon immunisation with Re cells, although *E. coli* K12Re, R1, O6, and O18 remained unaltered. Immunisation with Re resulted in IgG to all *S. minnesota* R-LPS becoming detectable at low levels, and large increases appearing against *S. typhimurium* Ra and Re LPS. Lipid A response was also boosted slightly. Rises from baseline levels to moderate levels were observed versus *P. aeruginosa* PAC605; *K. aerogenes* M10B; and *E. coli* J5 and O12.

TABLE 3:1. Immunogens for Longitudinal Study of Immunoglobulin Response to Lipopolysaccharides in Rabbits.

DAY	Rabbit 130	Rabbit 131	Rabbit 132	Rabbit 133	Rabbit 134	Rabbit 135
0	S. typhimurium lipid A	S. typhimurium R1542 Ra	S. typhimurium R878 Rc	E. coli F515 Re	E. coli O18	E. coli O18
28	S. typhimurium R1102 Re	S. typhimurium R119 Rb	E. coli J5 Rc	E. coli C62 Ra/b	E. coli O6	E. coli O18
56	S. typhimurium R1032 Rd	S. typhimurium R878 Rc	K. aerogenes M10B Rb	E. coli K12 Ra	E. coli O4	P. aeruginosa Habs type 1
84	S. typhimurium R878 Rc	S. typhimurium R1032 Rd	P. aeruginosa PAC605 Rc	deceased	E. coli O2	E. coli O6
112	S. typhimurium R119 Rb	S. typhimurium R1102 Re	E. coli F515 Re		E. coli O1	E. coli O18
140	S. typhimurium R1542 Ra	S. typhimurium lipid A	S. minnesota R595 Re		E. coli O16	K. aerogenes M10

FIGURES 3:11 to 3:15.
Key to immunogens and antigens.

Organism				ELISA antigen	Bacterial immunogen
S.	typhimurium		wild type	WTST	N/I**
"	"	"	R1542	RAST	S.TYPH 1542 Ra
"	"	"	R119	RBST	S.TYPH 119 Rb
"	"	"	R878	RCST	S.TYPH 878 Rc
"	"	"	R1032	RDST	S.TYPH 1032 Rd
"	"	"	R1102	REST	S.TYPH 1102 Re
"	"	"	lipid A	N/A*	S.TYPH LIPIDA
S.	minnesota		R60	RASM	N/I
"	"	"	R345	RBSM	N/I
"	"	"	R5	RCSM	N/I
"	"	"	R7	RDSM	N/I
"	"	"	R595	RESM	S.MINN R595 Re
"	"	"	lipid A	LASM	N/I
E.	coli		R1	ECR1	N/I
"	"	"	R2	ECR2	N/I
"	"	"	R3	ECR3	N/I
"	"	"	R4	ECR4	N/I
"	"	"	J5	ECJ5	E.COLI J5
"	"	"	F515	N/A	E.COLI F515
"	"	"	K12	ECK12	N/I
"	"	"	K12 Re	ECREK12	N/I
"	"	"	K12 lipid A	ECLAK12	N/I
"	"	"	01	N/A	E.COLI 001
"	"	"	02	ECO2	E.COLI 002
"	"	"	04	N/A	E.COLI 004
"	"	"	06	ECO6	E.COLI 006
"	"	"	012	ECO12	N/I
"	"	"	016	ECO16	E.COLI 016
"	"	"	018	ECO18	E.COLI 018
"	"	"	075	ECO75	N/I
"	"	"	086	ECO86	N/I
"	"	"	0111	ECO111	N/I
P.	aeruginosa		PAC605	PAC605	PS.AER PAC605
"	"	"	Habs type1	PAS1	PS.AER Habs-01
K.	aerogenes		M10B	KAM10B	K.AERM10B
"	"	"	M10	N/A	K.AERM10

N/A* : not used as antigen
N/I**: not used as immunogen

FIGURE 3:11a.
(Antigens and immunogens as described on facing page.)

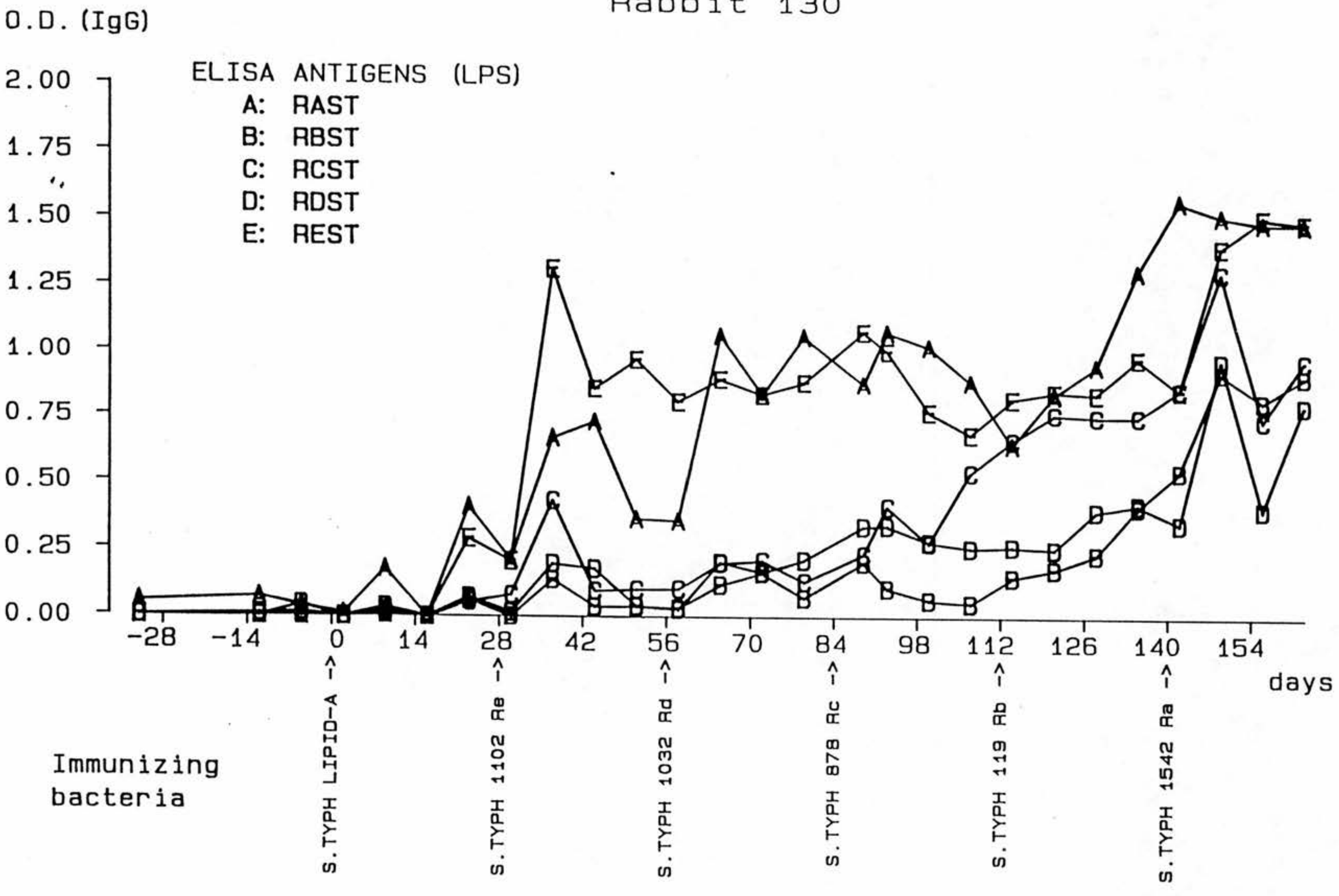


FIGURE 3:11b.
(Antigens and immunogens as described facing page 142.)

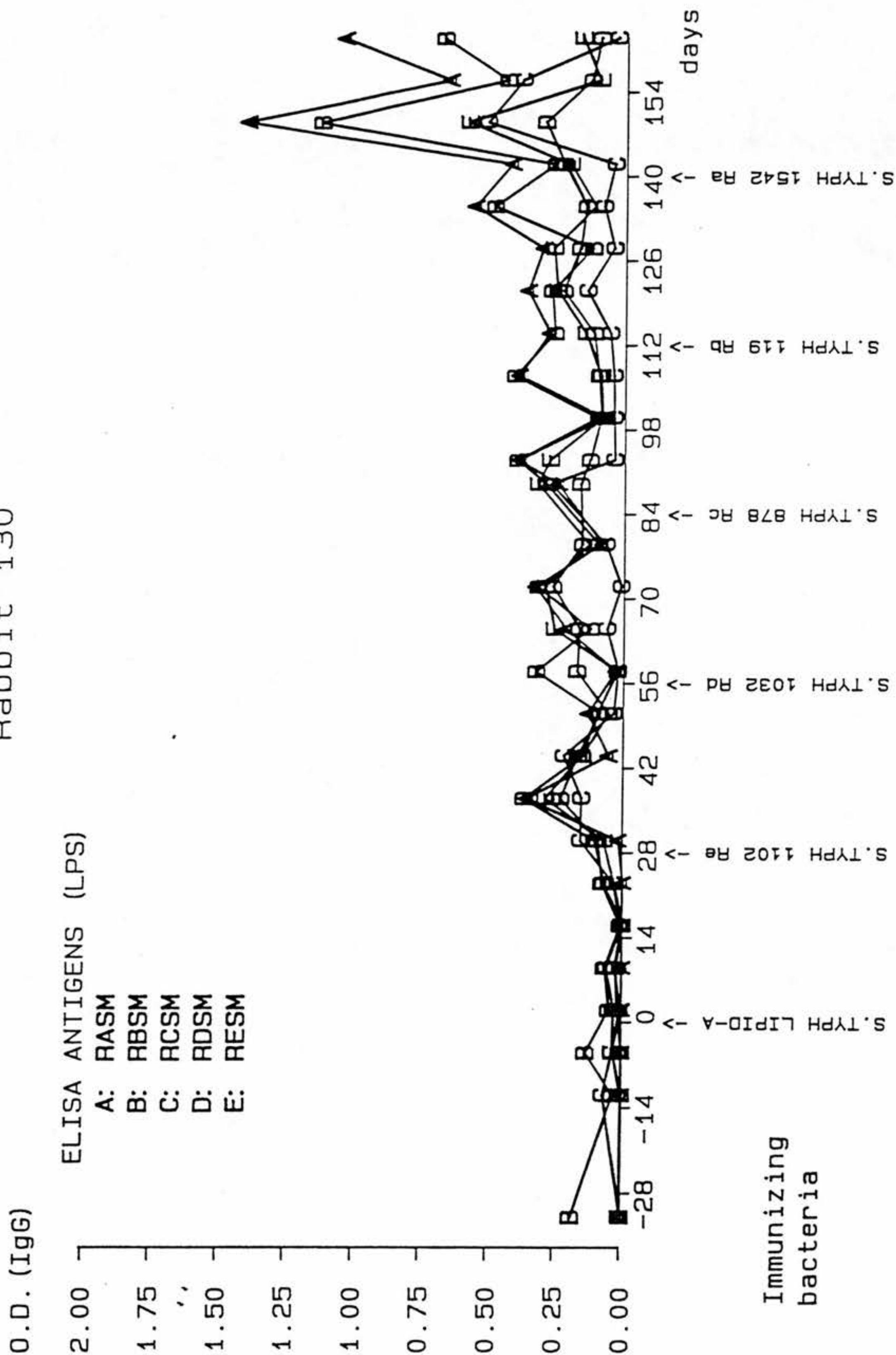


FIGURE 3:11c.
(Antigens and immunogens as described facing page 142.)

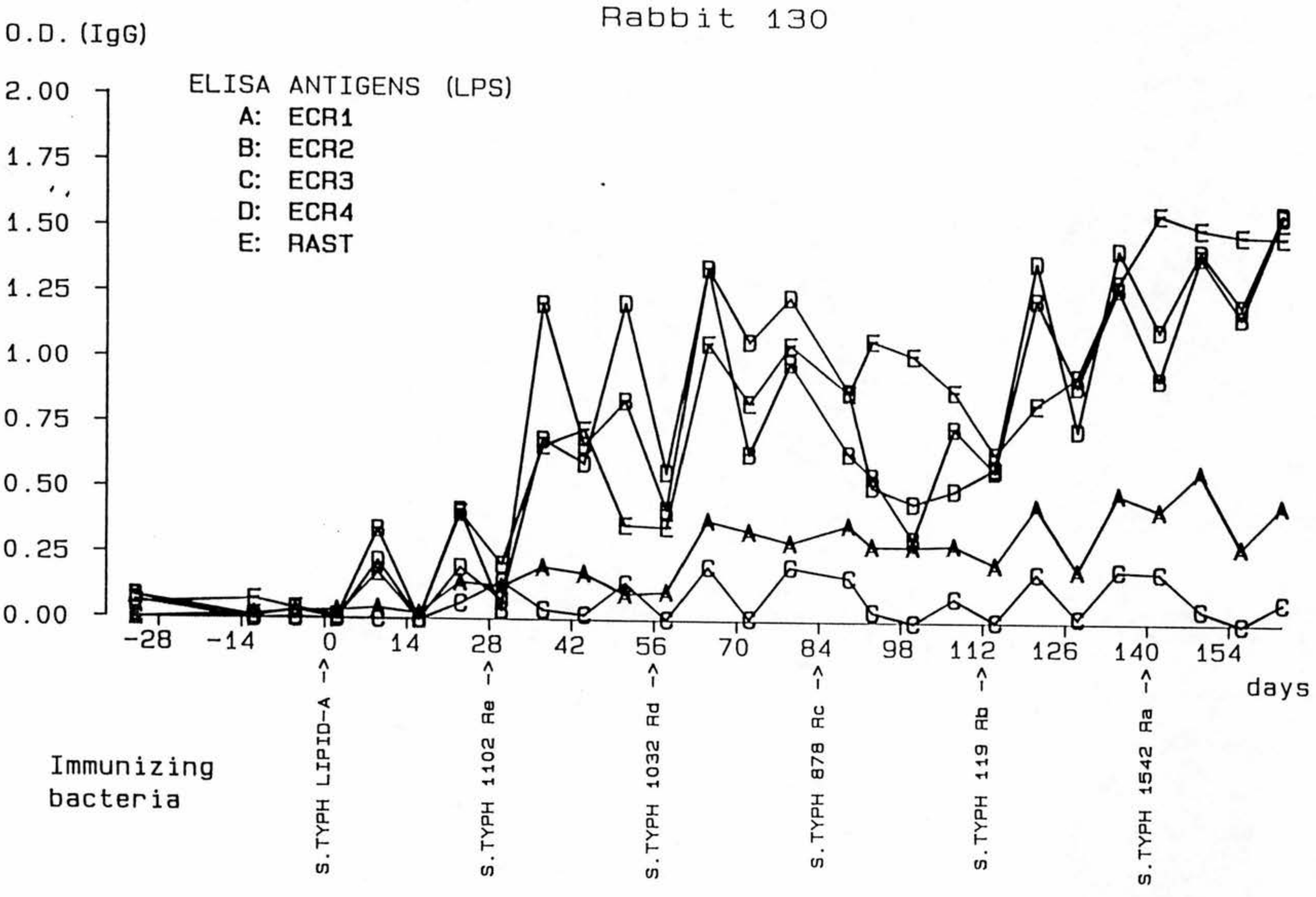


FIGURE 3:11d.
(Antigens and immunogens as described facing page 142.)

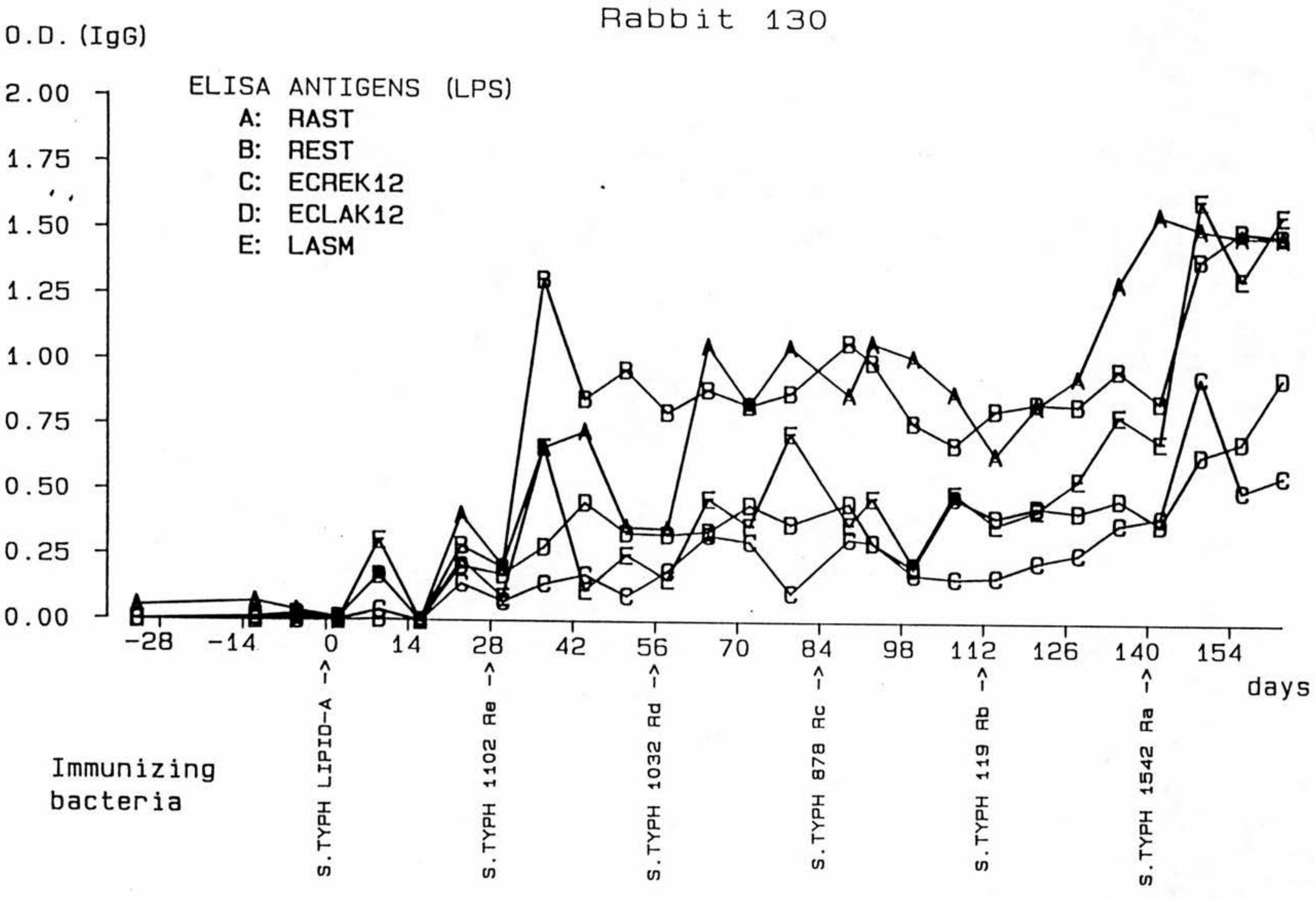


FIGURE 3:11e.
(Antigens and immunogens as described facing page 142.)

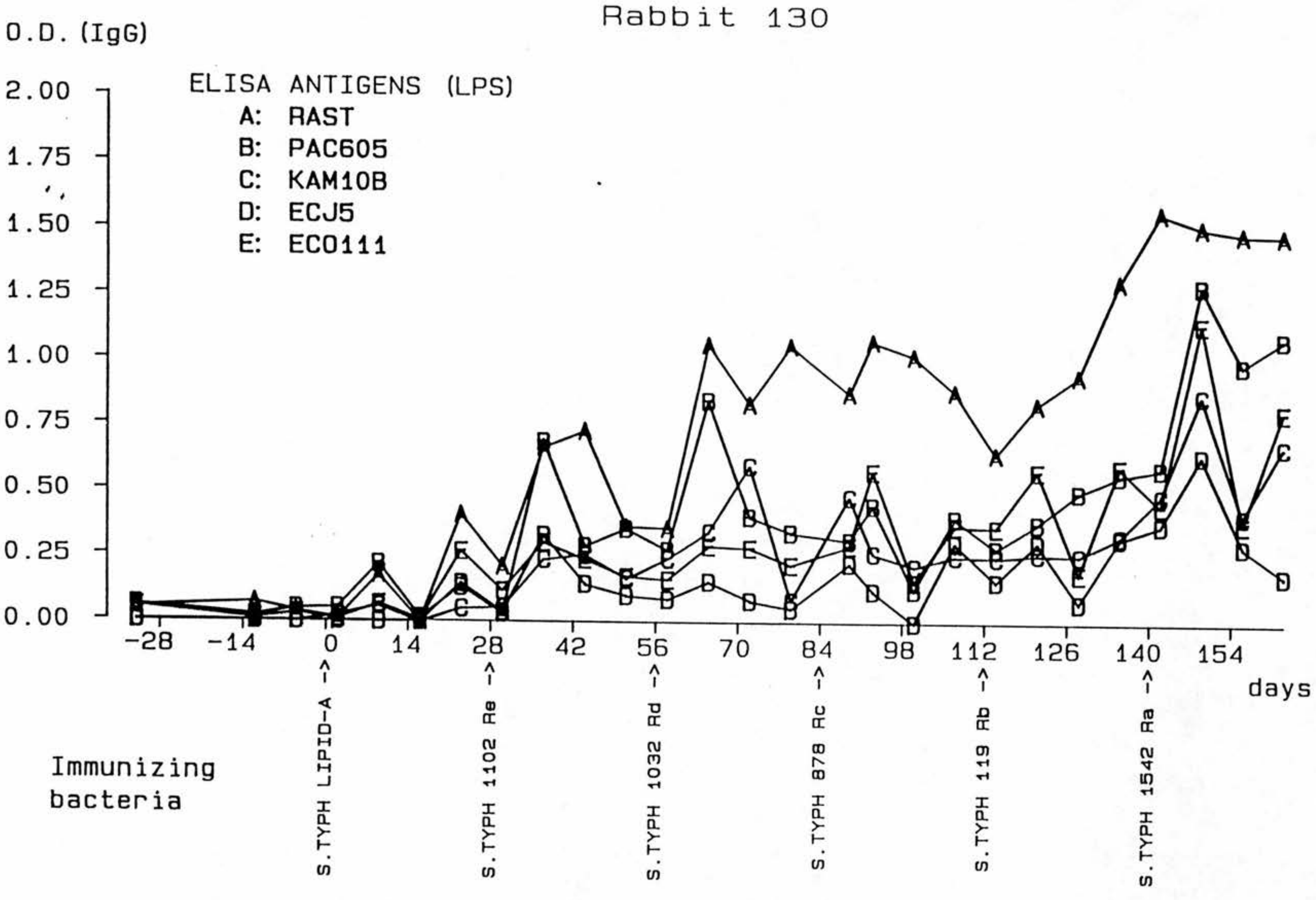


FIGURE 3:11f.
(Antigens and immunogens as described facing page 142.)

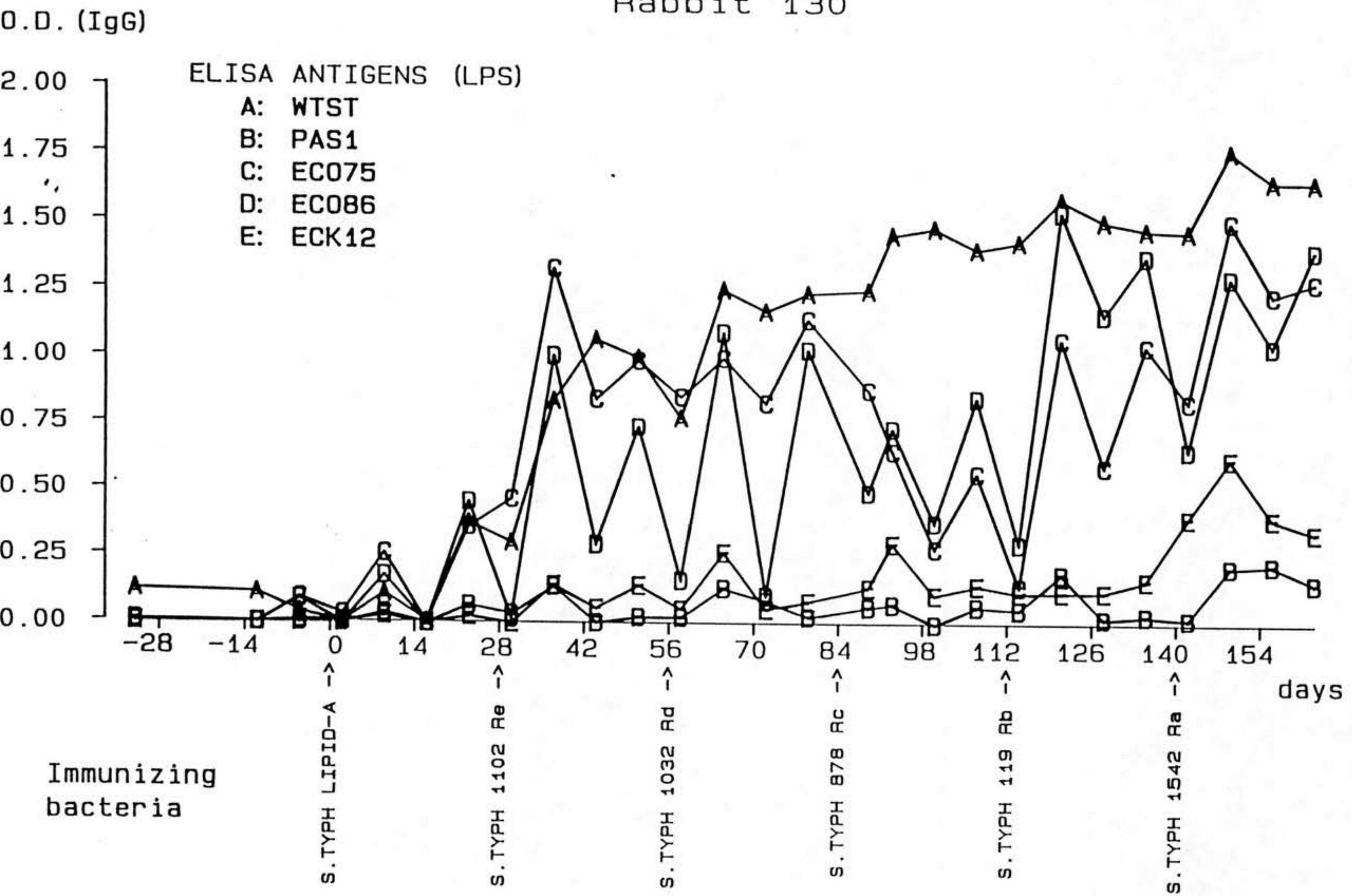
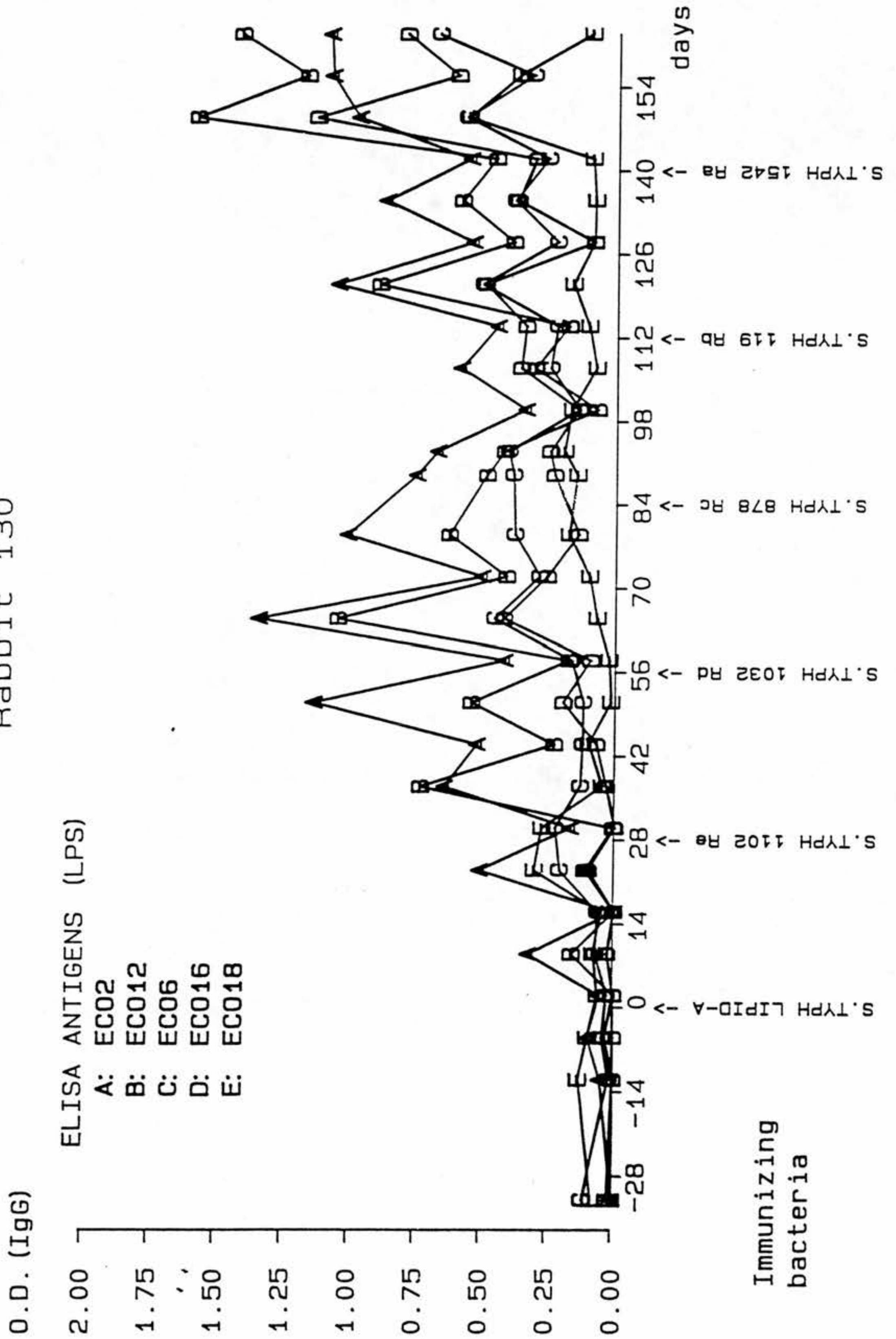


FIGURE 3:11g.
(Antigens and immunogens as described facing page 142.)



The next immunisation (with Rd) produced only a slight homologous response. No alteration was caused versus E. coli LPS from J5, R3, K12 lipid A and O111, while IgG versus O2, O12, and O18 IgG fell. A minor response was obtained to S. minnesota R-LPS, and modest to significant increases in absorbance were seen to all other antigens. S. typhimurium Rc produced an increase versus homologous antigen and against the parental O-antigen containing strain from low to moderate levels. Transient rises were induced versus E. coli O111 and K. pneumoniae M10B. Other antigens developed little response, and for many a declining trend was obtained.

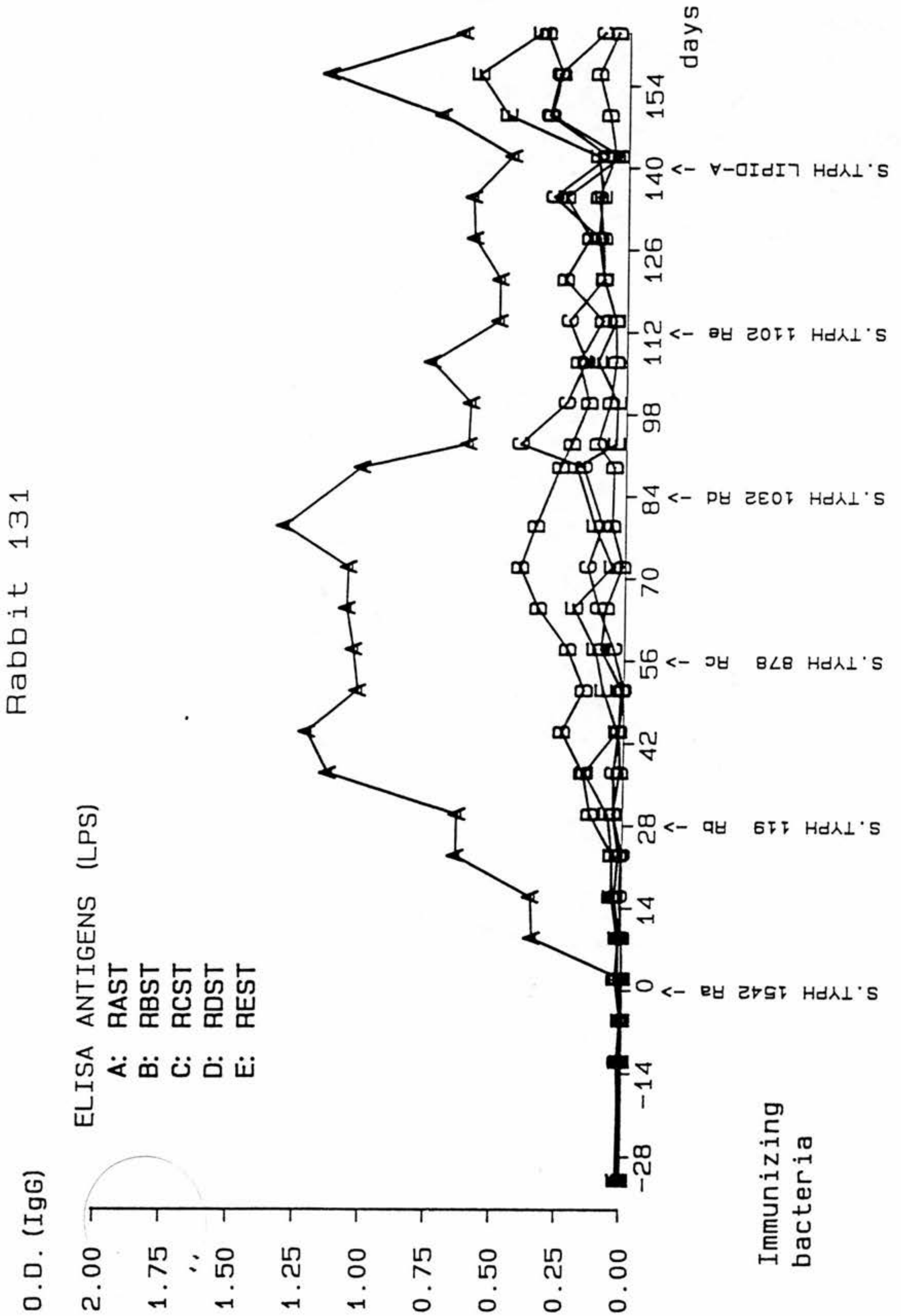
Largest responses produced by immunisation with Rb were against S. typhimurium Ra and E. coli O75 and O86, with a smaller rise in homologous IgG from low to moderate levels. Similar modest rises were seen to E. coli K12Re and R1; S. typhimurium Rc, Rd and Re; and to all S. minnesota preparations. Large fluctuations were observed for E. coli O2, O12, O16, O75, O86, O111, R2, and R4, though the last two showed a rising trend.

The final immunisation with Ra bacteria produced no homologous response. Little change was obtained versus S. typhimurium Rd, P. aeruginosa Habs type 1, or E. coli R1 and R3. Moderate to large responses were seen versus all other antigens, although versus four E. coli (J5, K12, O18, and O111), three S. minnesota mutants (Rb, Rc, and Re) and K. aerogenes M10B this was followed by a fall to pre-Ra immunisation levels.

3:3:2. Rabbit 131.

This animal was also immunised with a series of rough heat-killed S. typhimurium bacteria, but in descending size from Ra chemotype to lipid A. Figure 3:12a-g shows the response of IgG antibodies versus

FIGURE 3:12a.
(Antigens and immunogens as described facing page 142.)



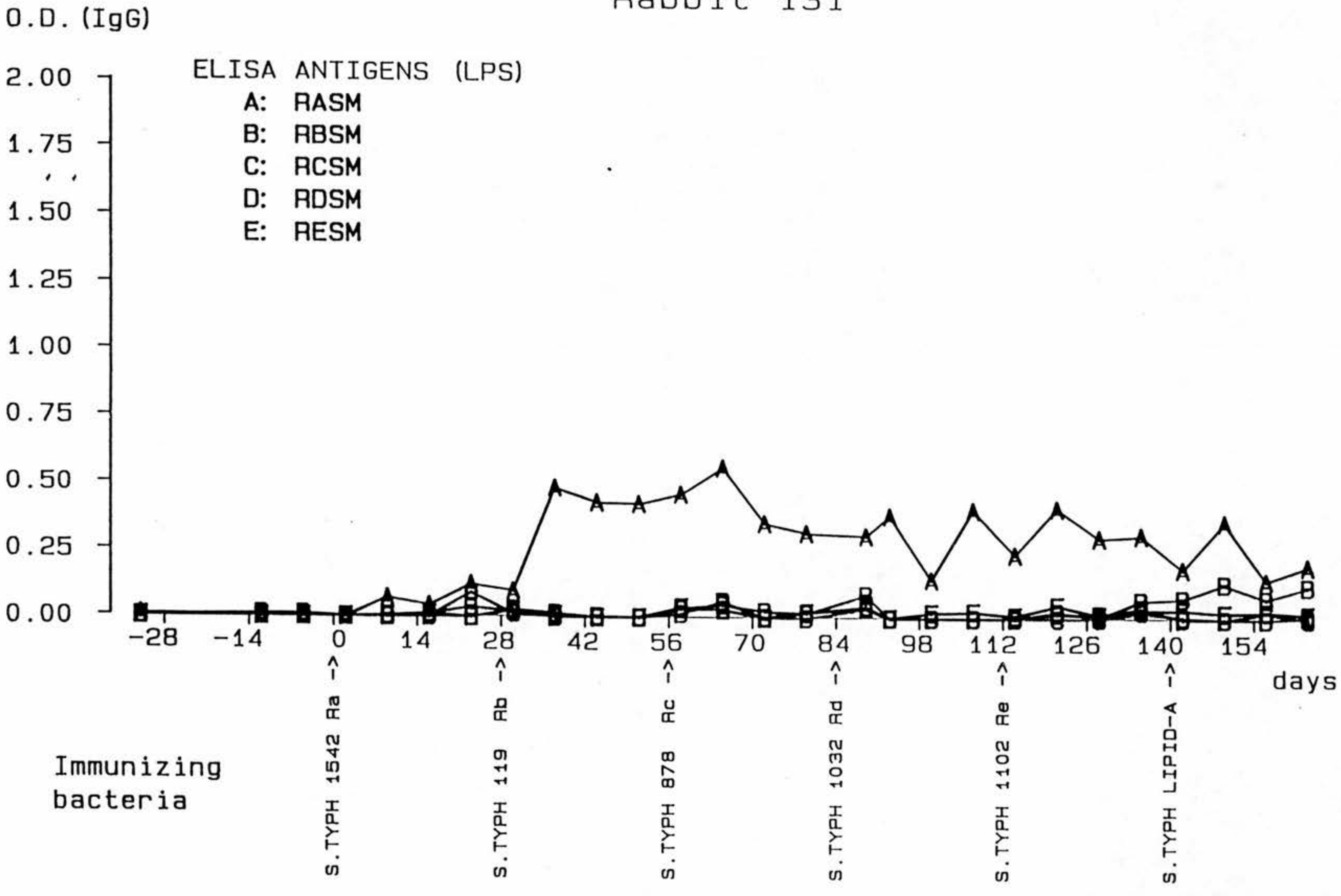


FIGURE 3:12b.
(Antigens and immunogens as described facing page 142.)

FIGURE 3:12c.
(Antigens and immunogens as described facing page 142.)

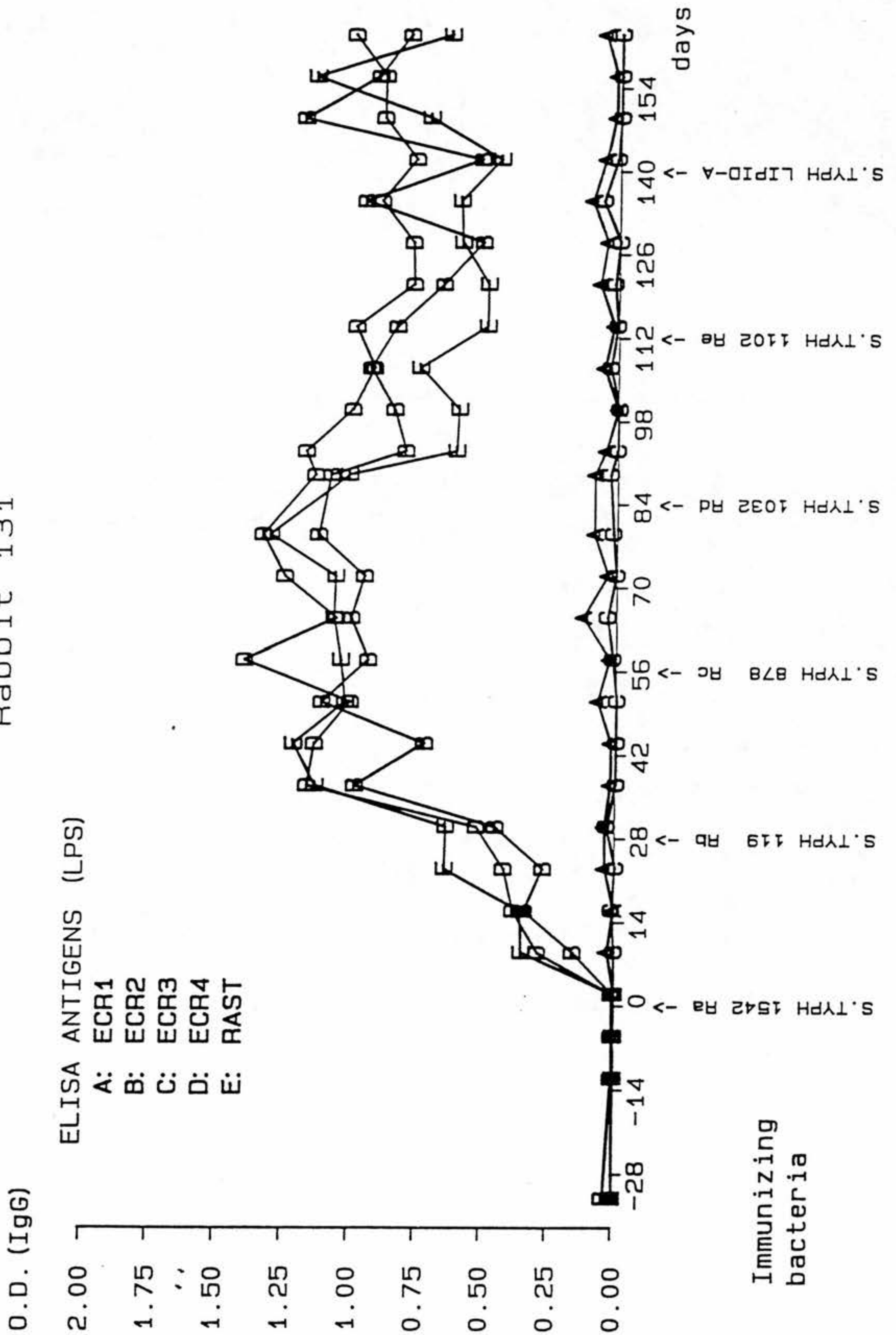
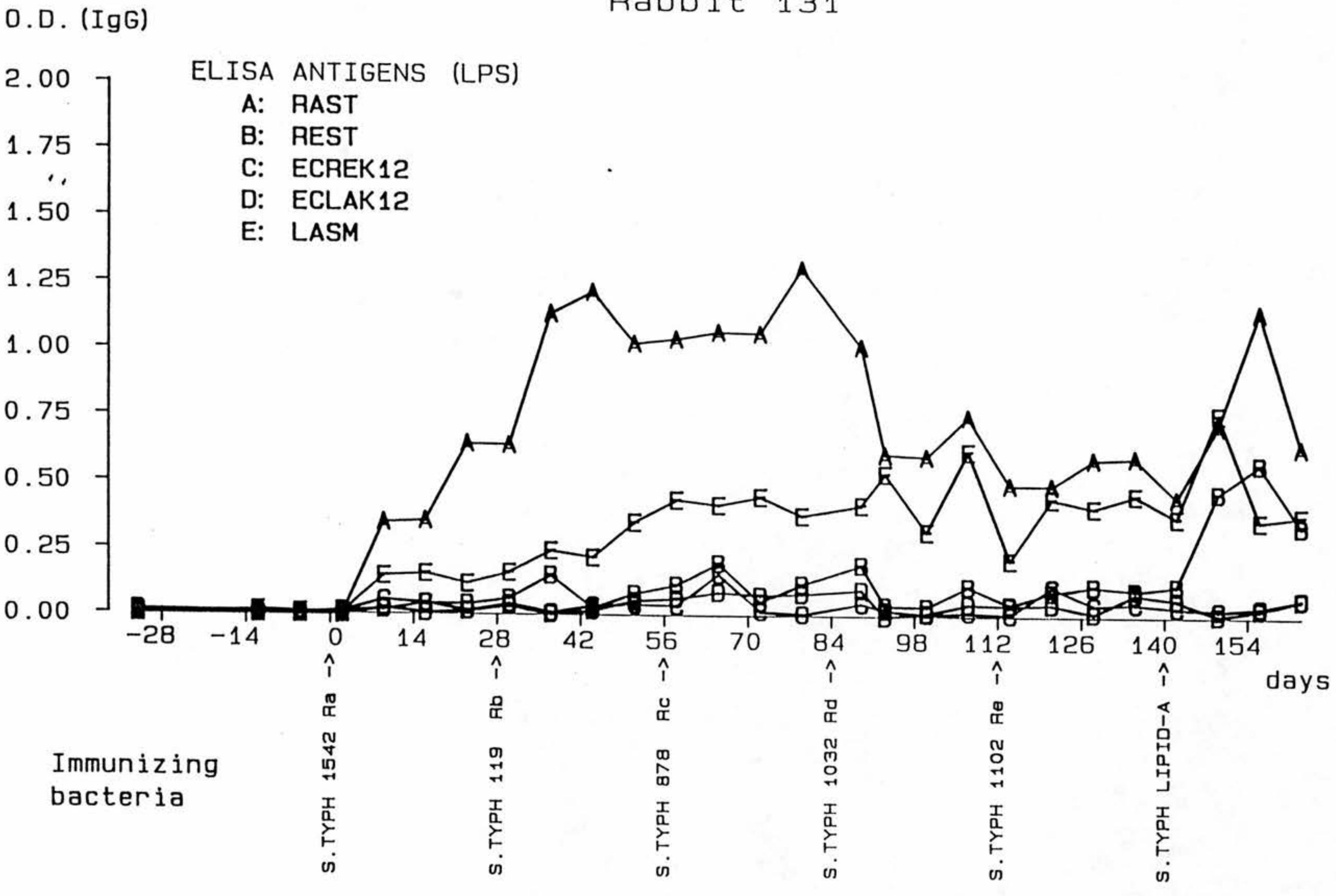


FIGURE 3:12d.
(Antigens and immunogens as described facing page 142.)



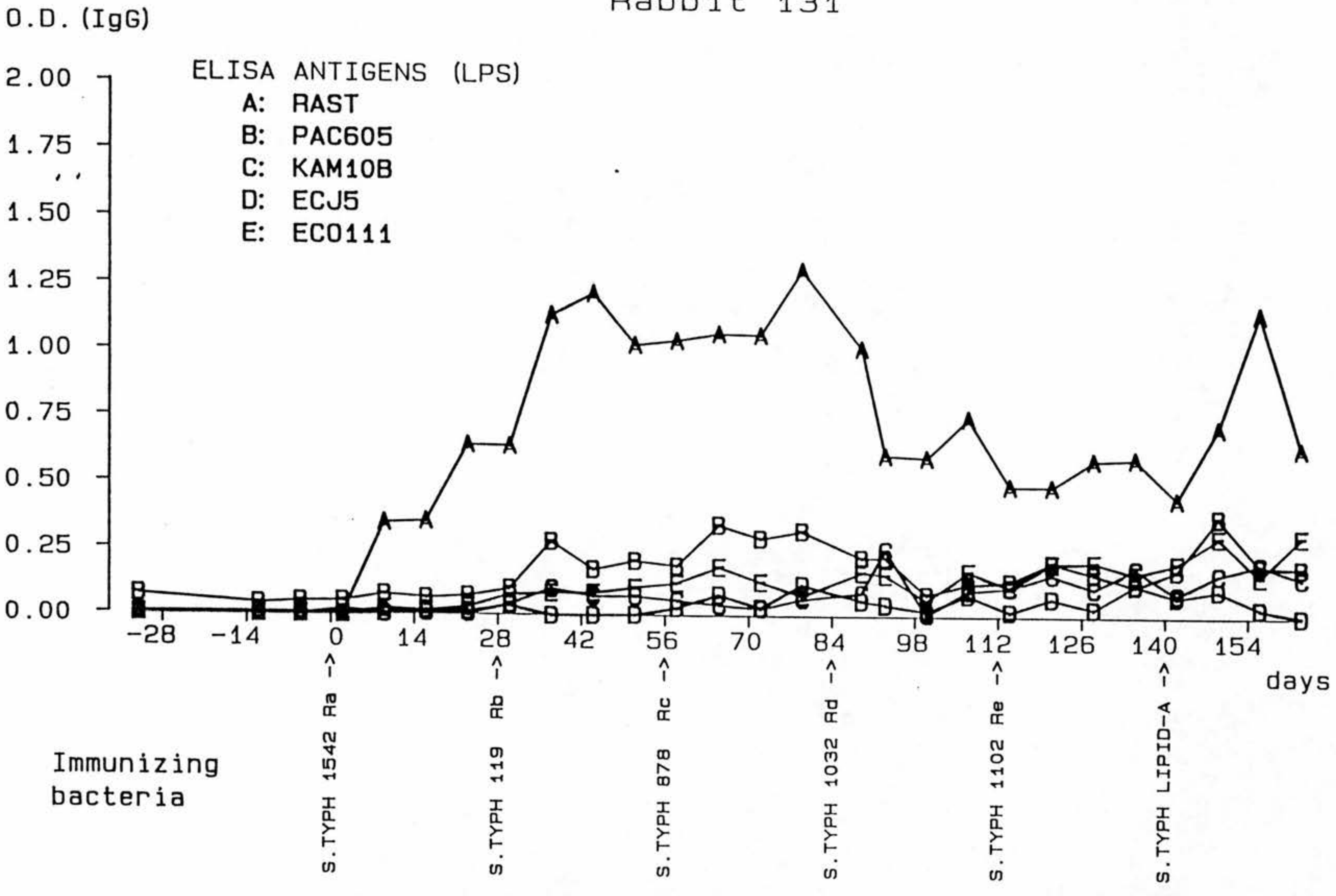


FIGURE 3:12e.
(Antigens and immunogens as described facing page 142.)

FIGURE 3:12f.
(Antigens and immunogens as described facing page 142.)

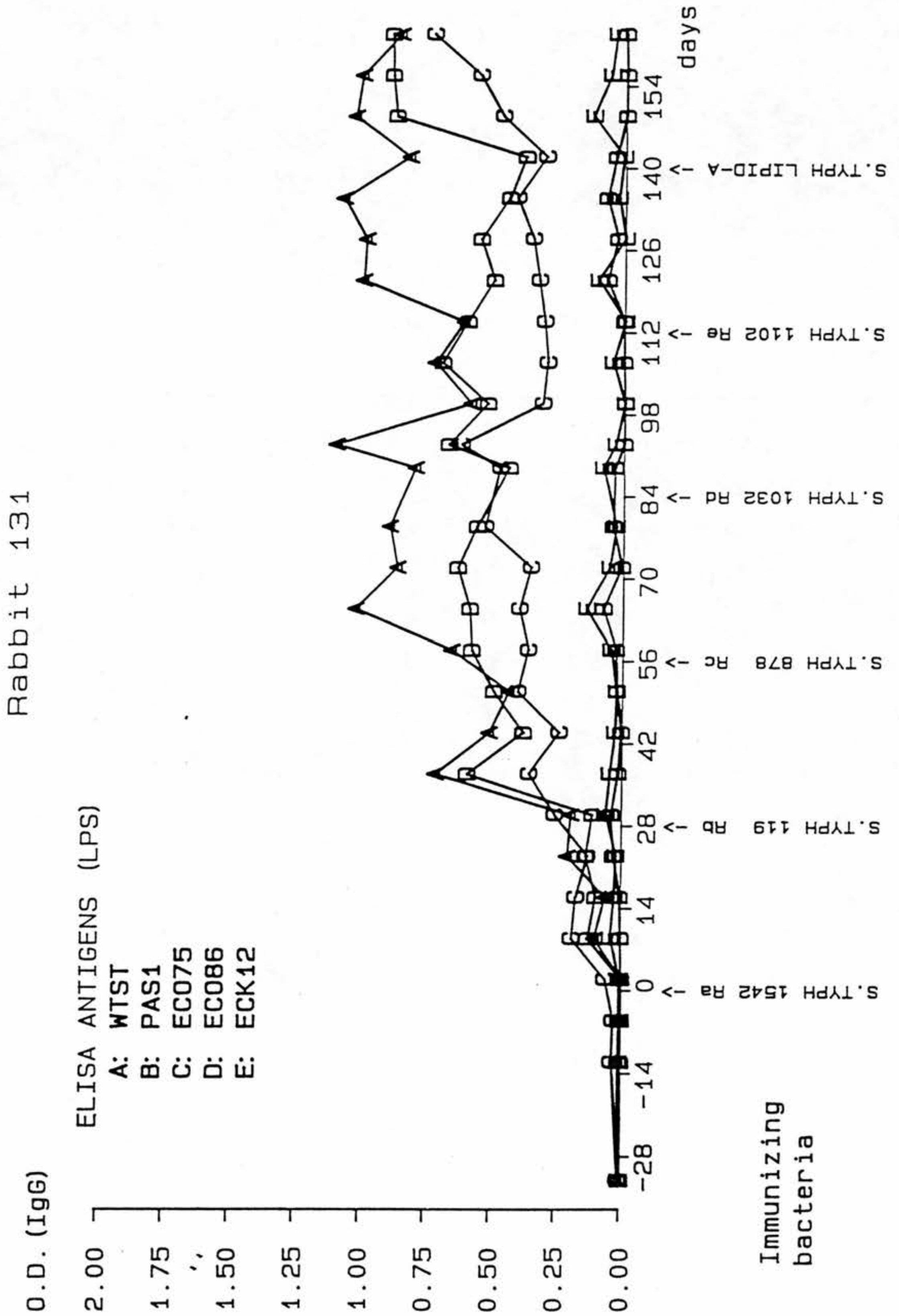
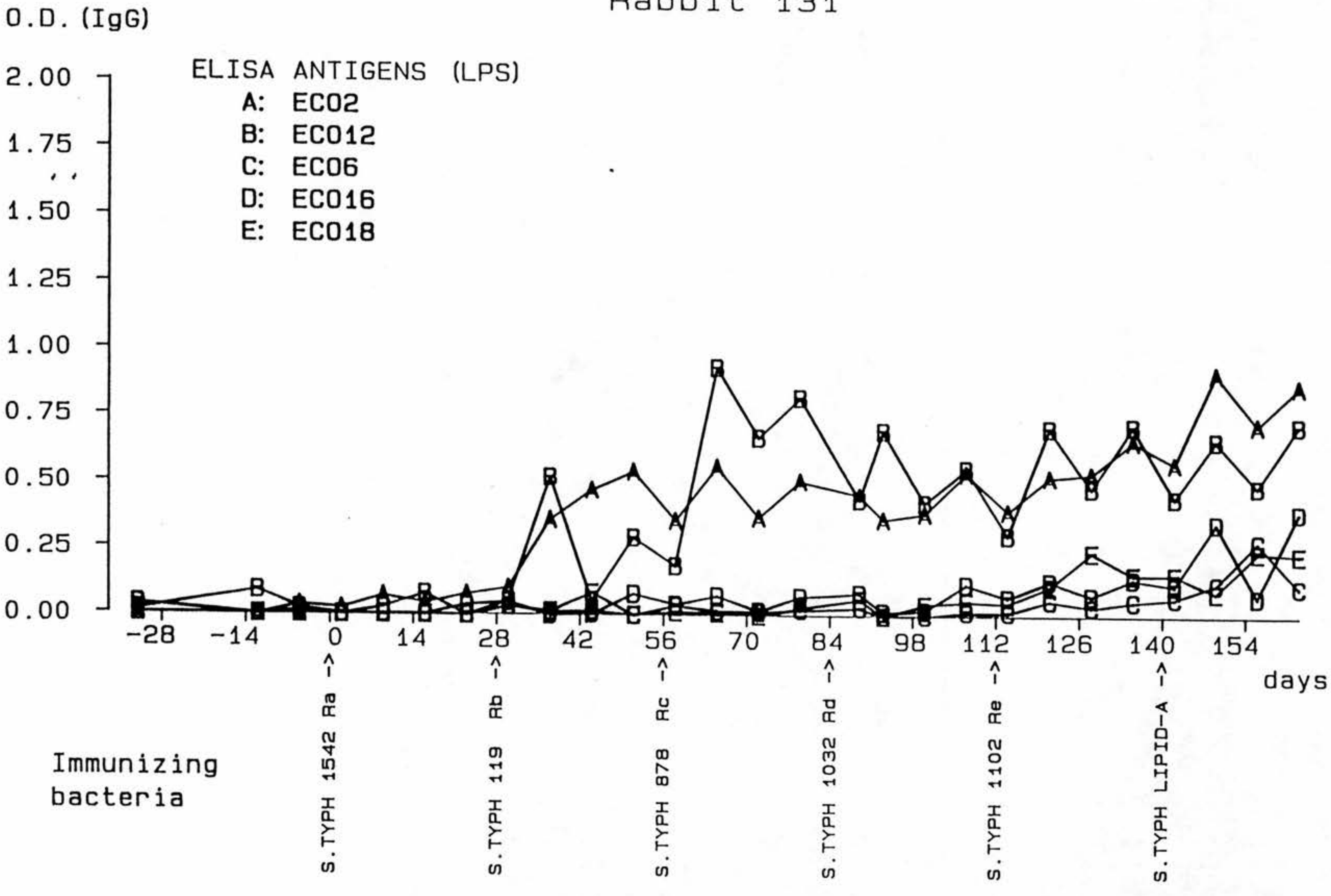


FIGURE 3:12g.
(Antigens and immunogens as described facing page 142.)



the LPS antigens in ELISA.

Prior to immunisation, levels of IgG were negligible or not detectable. Immunisation on day zero produced a marked rise in IgG titre to the homologous antigen (S. typhimurium Ra) and to E. coli R2 and R4. Modest rises to S. typhimurium wild type, S. minnesota lipid A, and E. coli 075 and 086 were also produced. Minimal response was obtained to other antigens.

Immunisation with Rb boosted all IgG induced previously, particularly large rises being seen to S. typhimurium wild type and Ra, and to E. coli 086, R2, and R4. Smaller responses were obtained to the homologous antigen, S. minnesota lipidA, and to E. coli 075. Initial responses were obtained to several antigens: those to S. minnesota Ra and P. aeruginosa PAC605 were modest while those versus E. coli 02 and 012 were large.

Rc LPS from S. typhimurium produced only small rises to homologous LPS and to S. typhimurium Rb and E. coli 075, with larger increments to S. typhimurium wild type and to P. aeruginosa PAC605. IgG to other antigens remained reasonably stable.

After challenge of rabbits with Rd there was a sharp fall in IgG to S. typhimurium Ra, P. aeruginosa PAC605, and to E. coli 012, R2 and R4. Modest increases followed by return to pre-Rd immunisation levels occurred with S. typhimurium Rc, E. coli 075, and K. aerogenes M10B. Once again IgG to the remaining LPS remained fairly stable.

Heat-killed S. typhimurium Re produced modest rises to S. minnesota lipid A and E. coli 02, 012, and 018. Activity of IgG against E. coli R2 and R4 continued to decline slowly as did that against strain 086.

The final immunisation resulted in moderate to large increases in IgG versus S. typhimurium R-LPS except Rd which did not alter. Similarly, IgG to E. coli O75 and O86 were obtained. Activity directed towards E. coli R2 and R4, S. minnesota lipid A, and P. aeruginosa PAC605 increased, but then returned to levels found prior to this immunisation. Large fluctuations were observed in IgG to the remaining E. coli O-types, though an underlying upwards trend was observed. All other IgG levels remained stable.

3:3:3. Rabbit 132.

Although most IgG levels (figure 3:13a-g) were negligible, low levels of IgG to S. minnesota Ra and Rb, S. typhimurium wild type, P. aeruginosa PAC605, and to E. coli O75 and O86 were observed to be present before the initial immunisation. This rabbit was immunised with a range of rough type organisms initially of Rc chemotype followed by Re chemotype.

The initial immunisation with S. typhimurium Rc produced modest increases in S. typhimurium and S. minnesota Ra lipopolysaccharides, and to those from E. coli J5, R1, R3, O6, O12, and O111. Larger increment were observed versus S. typhimurium Rc and wild type, and E. coli R2, R4, O2 and O86.

E. coli J5 mutant resulted in sharp rises to S. typhimurium Rc, S. typhimurium wild type, and to six E. coli LPS (R3, R4, O2, O6, O75, and O86). A small, but transient, rise was seen to the homologous antigen. Similar small, though persistent, increases were obtained to S. minnesota Rc and lipid A, S. typhimurium Re, P. aeruginosa PAC605, K. aerogenes M10B, and E. coli R1, R2, K12, O16 and O18.

The rough mutant M10B from K. aerogenes induced steep rises in IgG

FIGURE 3:13a.
(Antigens and immunogens as described facing page 142.)

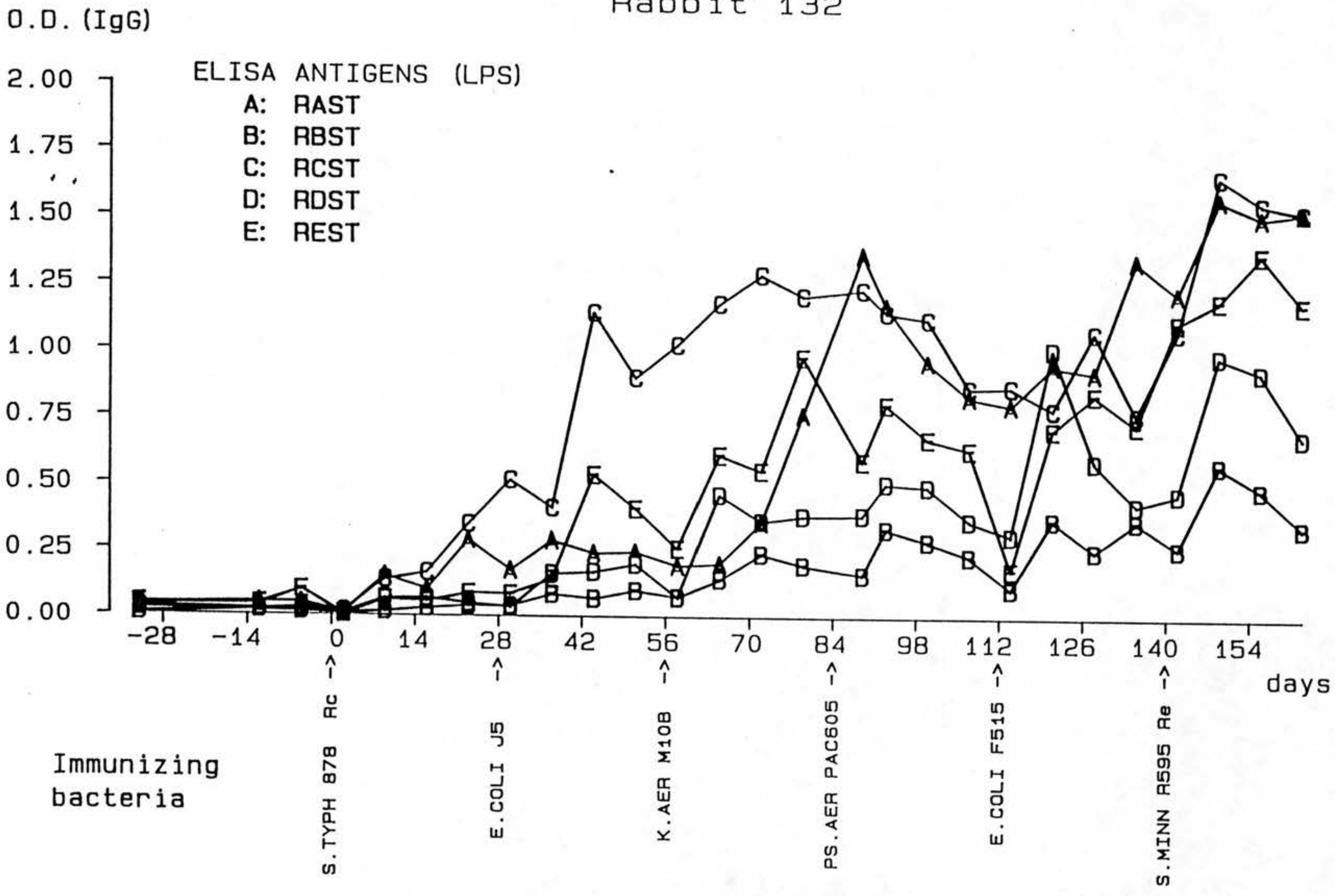


FIGURE 3:13b.
(Antigens and immunogens as described facing page 142.)

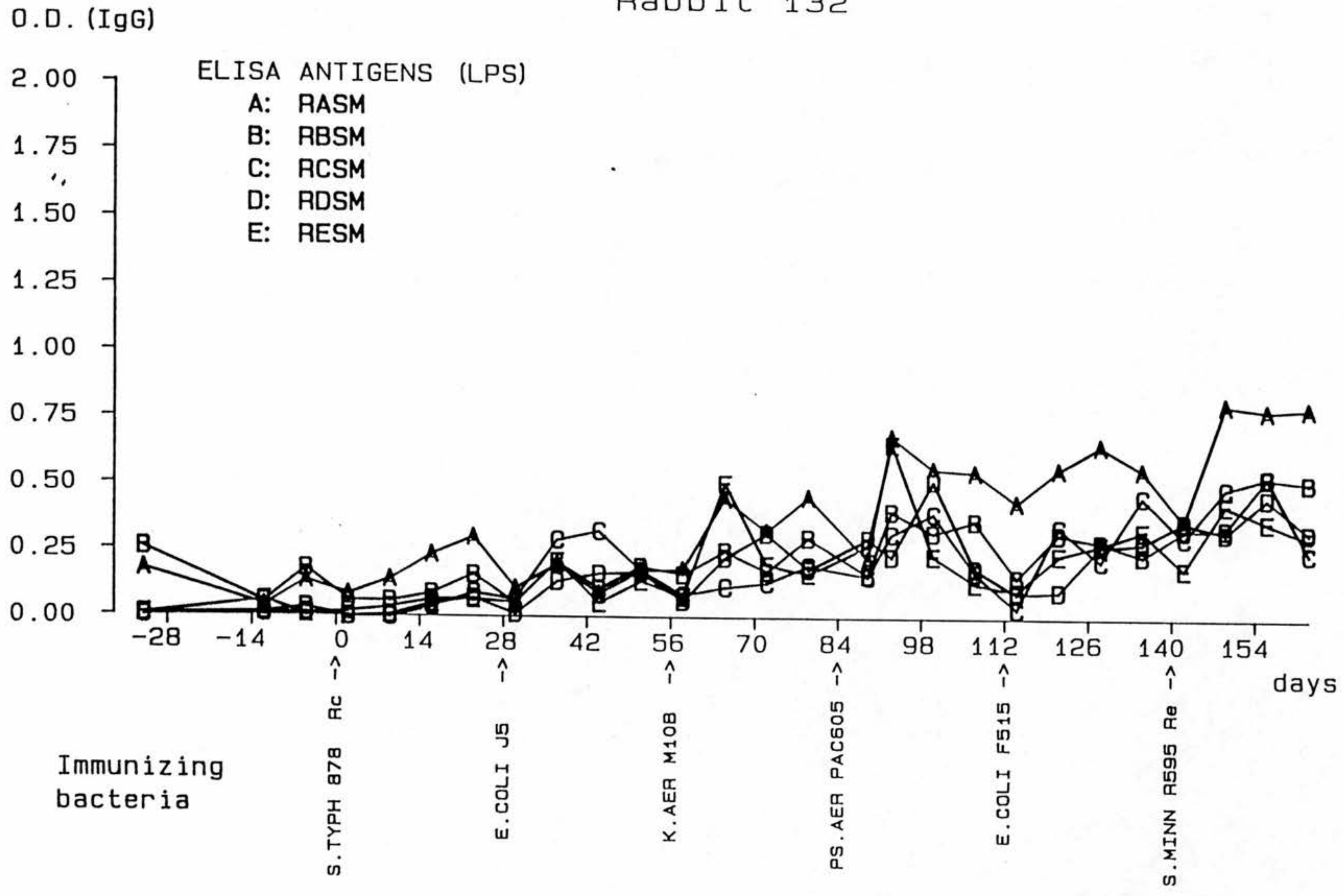


FIGURE 3:13c.
(Antigens and immunogens as described facing page 142.)

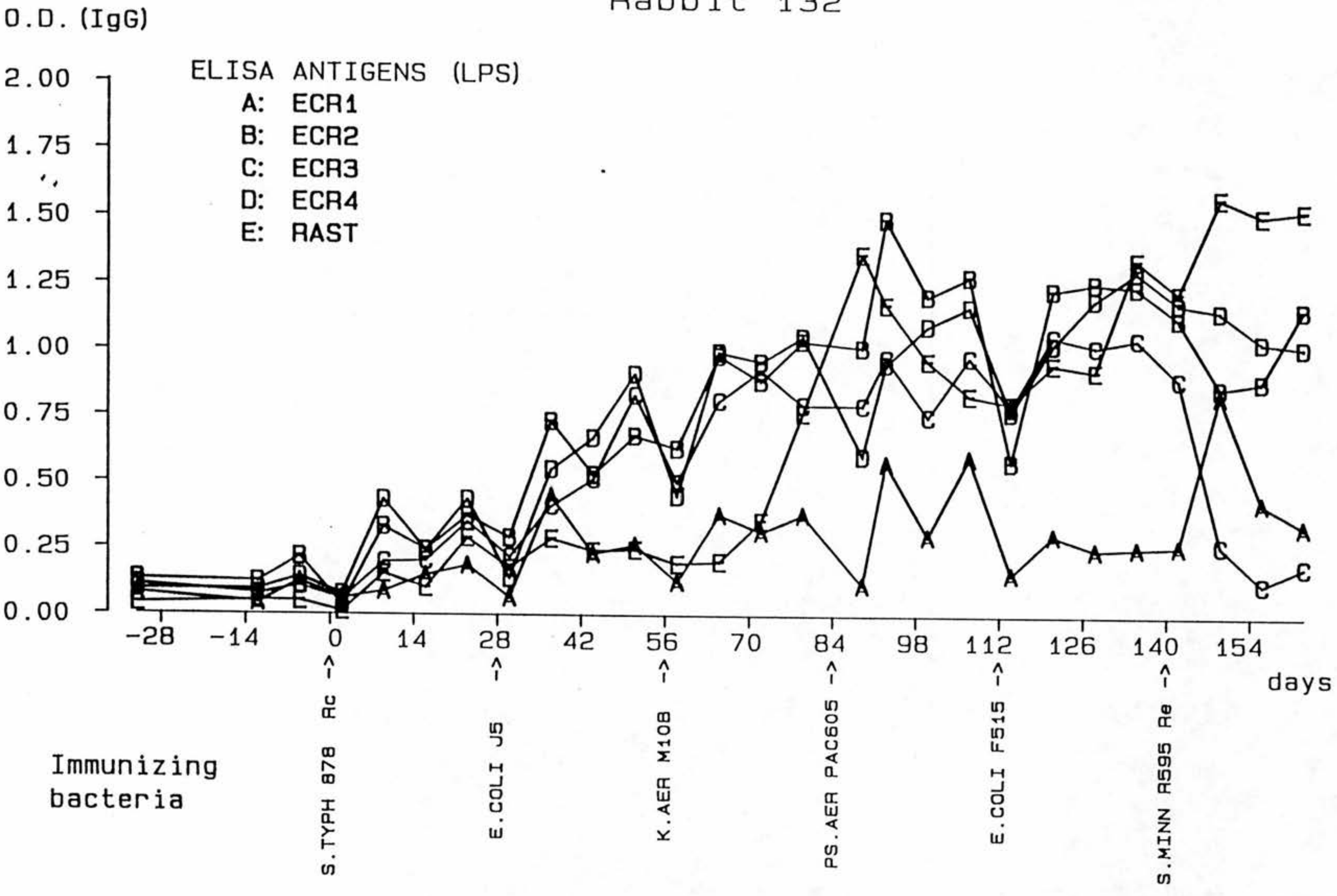


FIGURE 3:13d.
(Antigens and immunogens as described facing page 142.)

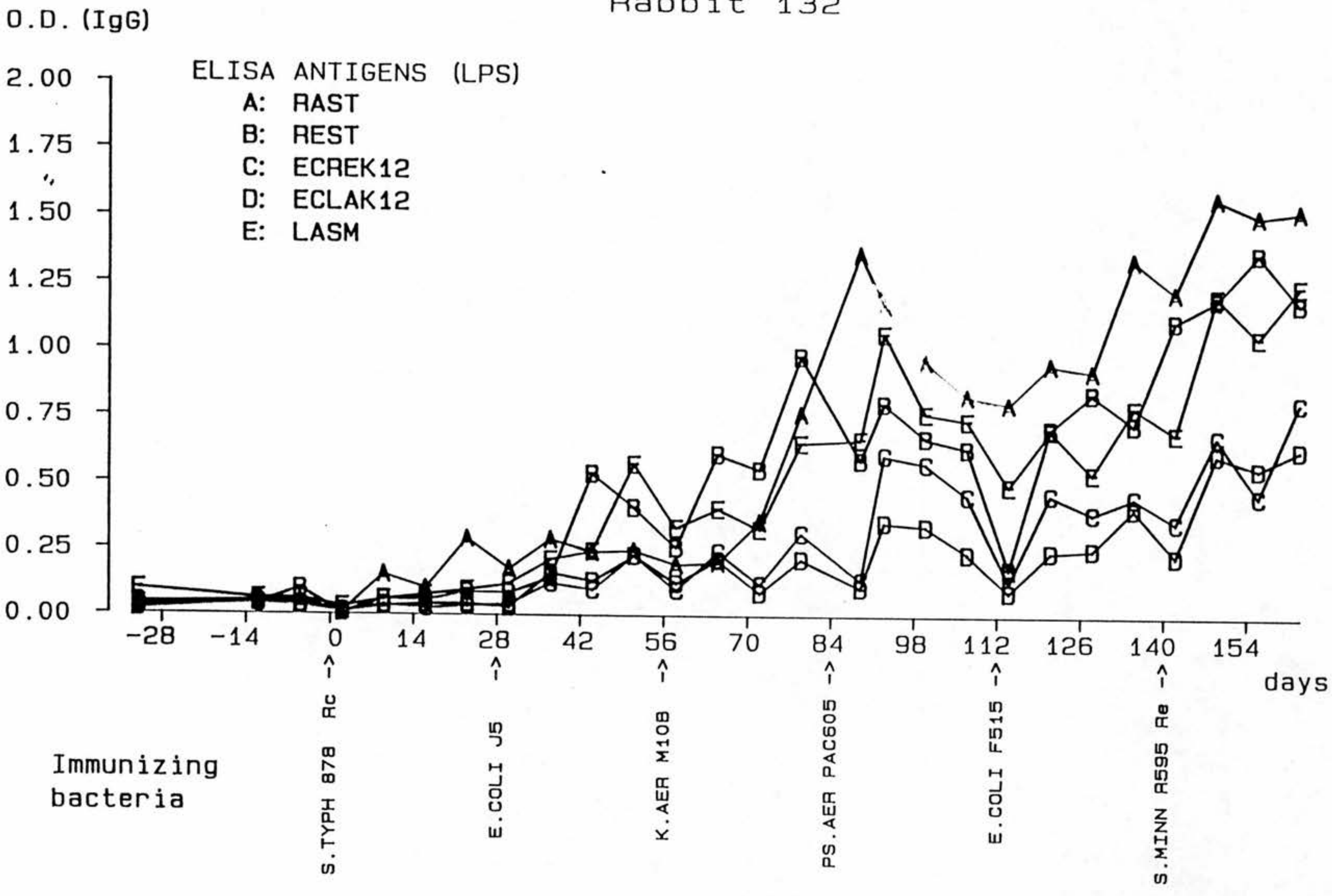


FIGURE 3:13e.
(Antigens and immunogens as described facing page 142.)

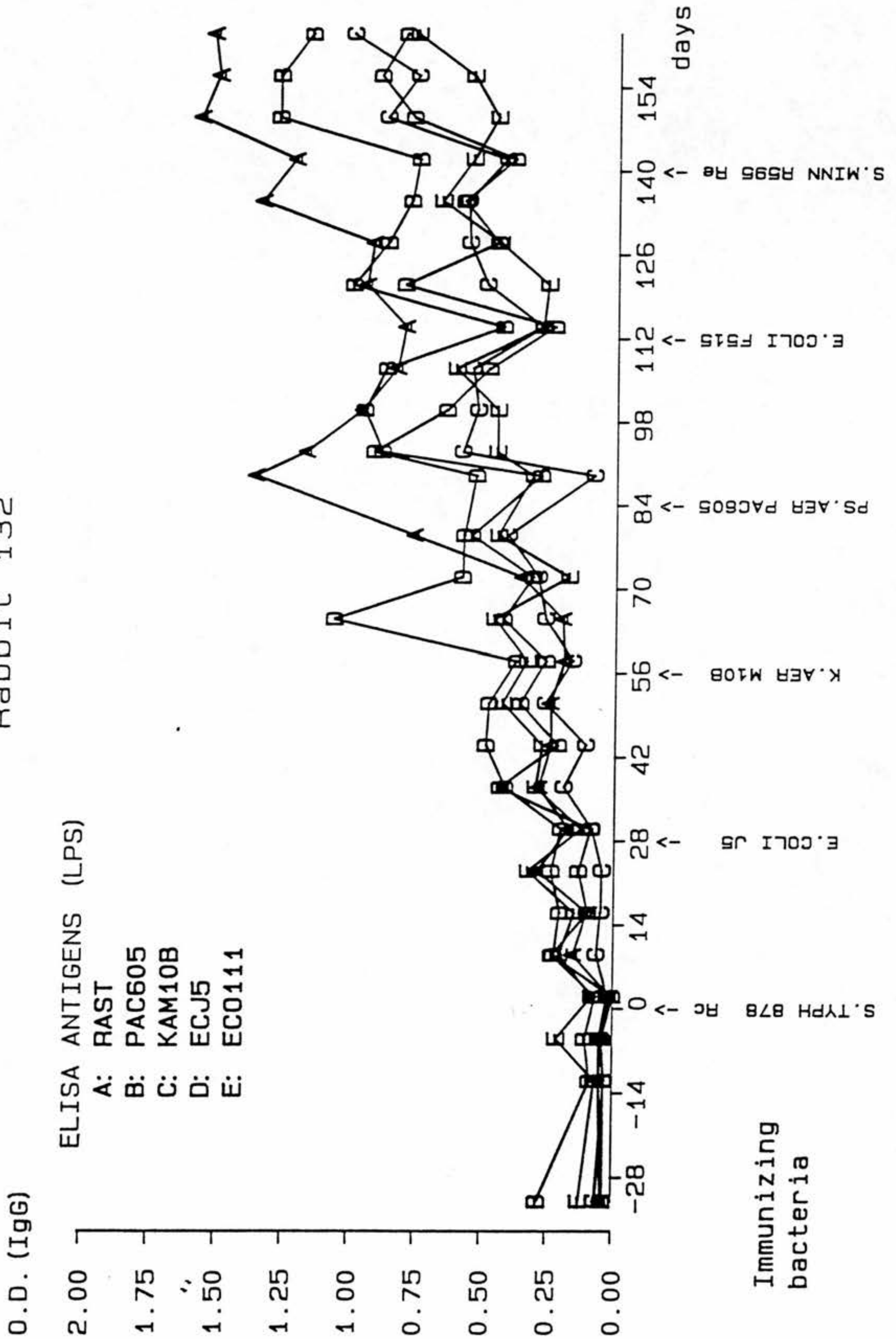


FIGURE 3:13f.
(Antigens and immunogens as described facing page 142.)

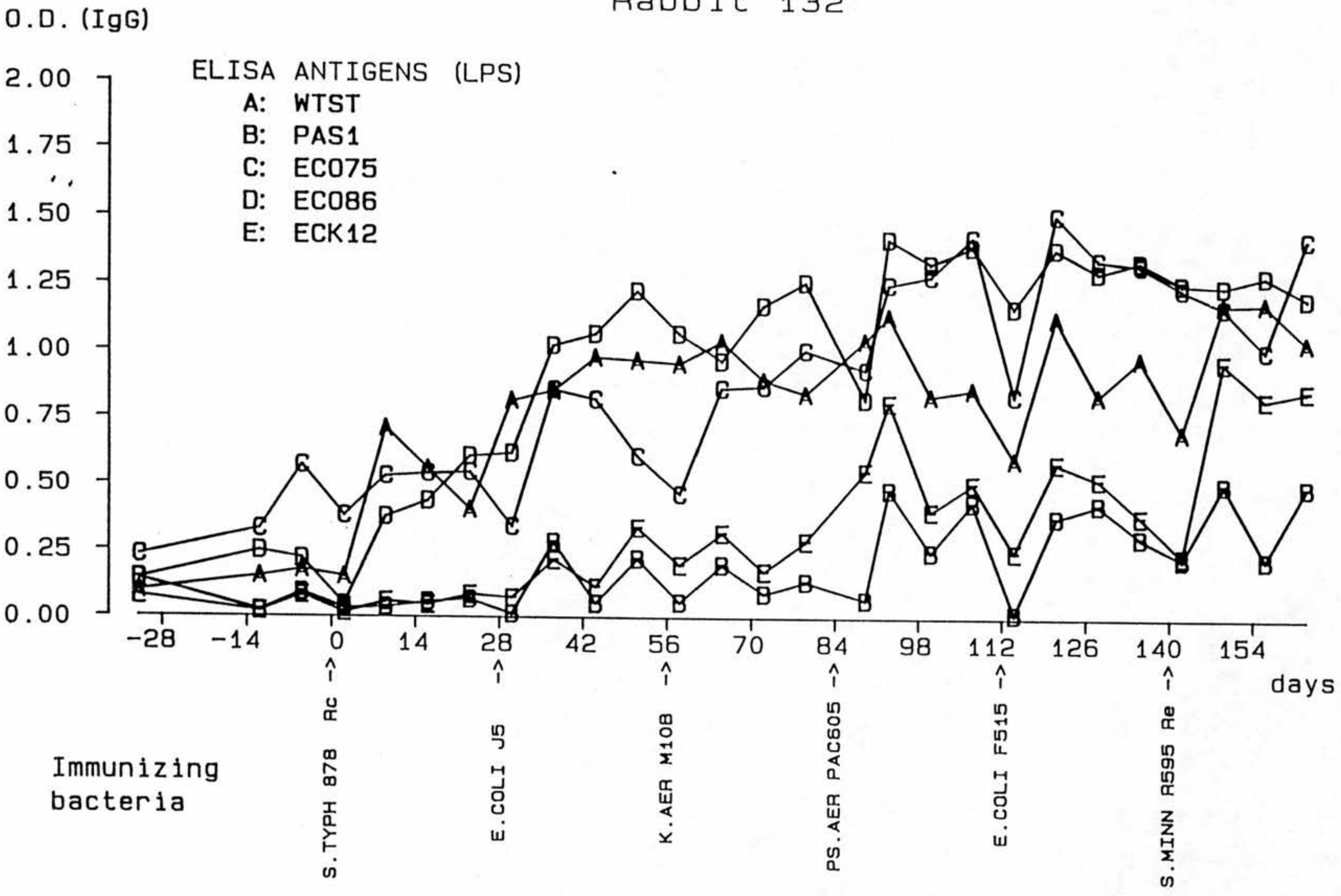
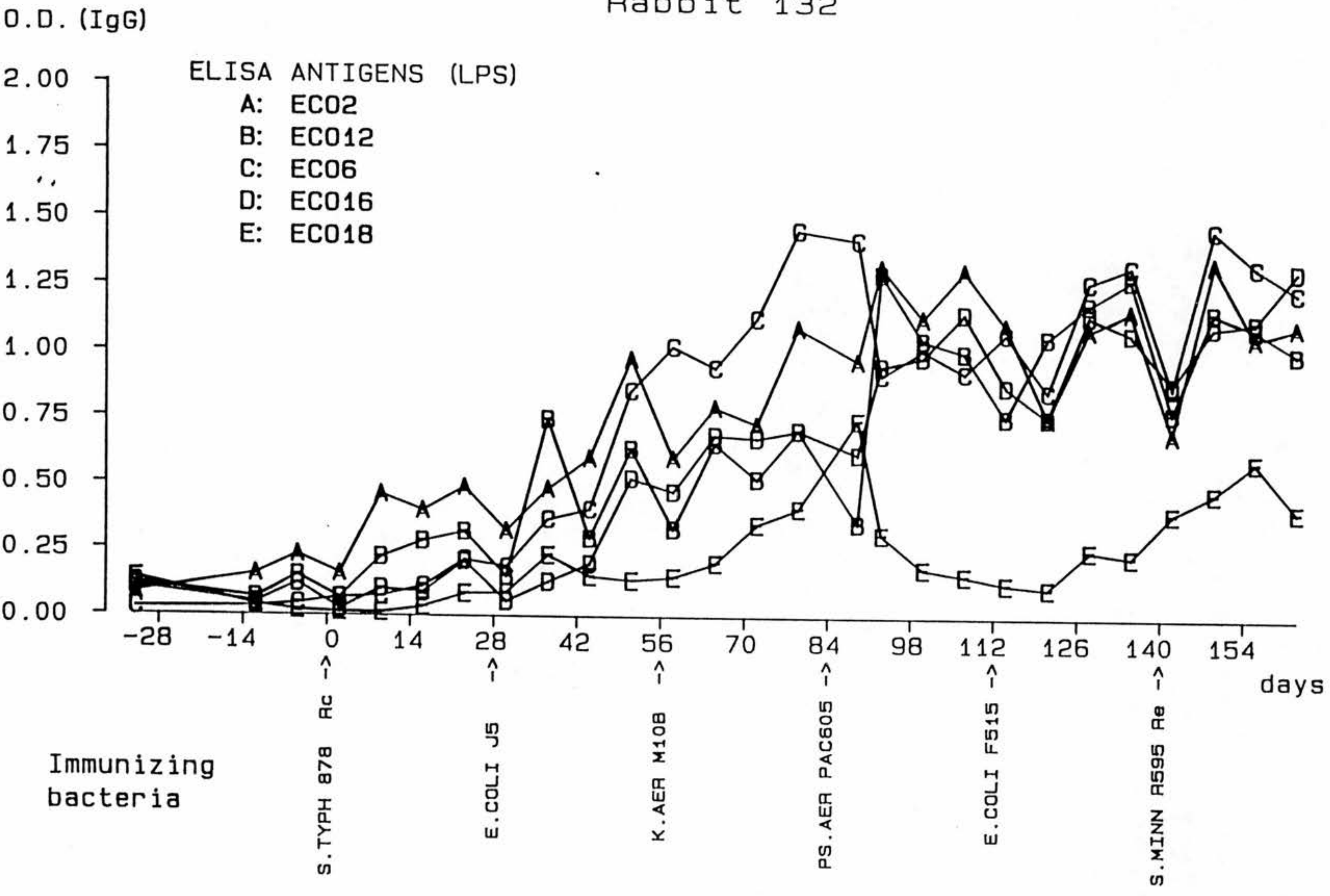


FIGURE 3:13g.
(Antigens and immunogens as described facing page 142.)



to S. typhimurium Ra and Re, and to E. coli J5, 06, 018 and 075, though the rise to Ra and 018 were delayed. Moderate rises were observed to the M10B itself, to S. typhimurium Rc and Rd, and to E. coli R1, R2, R3, R4, and 086.

P. aeruginosa PAC605 produced transient rises to S. typhimurium Ra and wild type, to S. minnesota Re and lipid A, and to E. coli J5, K12, K12Re, K12 lipid A, 012 and 018, although levels remained moderate after falling. IgG directed towards E. coli 02 and R3, the remaining S. typhimurium, and S. minnesota Rb, Rc, and Rd LPS were unchanged. The remaining LPS, including that from the immunising strain, showed more persistent increases.

Re type LPS from E. coli F515 produced little change in IgG levels to P. aeruginosa Habs type 1 and to E. coli K12 and 086 after an initial drop. Many other LPS showed increases in reactivity varying from moderate (S. typhimurium wild type, Rb and Rc, all S. minnesota antigens, P. aeruginosa PAC605, and E. coli J5, R3, K12, K12Re, K12 lipid A, 02, 06, 016, and 018); to large (S. typhimurium Ra, Rd and Re, and E. coli R2 and 075). Those versus S. typhimurium Rd and wild type, P. aeruginosa PAC605, and E. coli J5, K12, 02, 06, 012, and 016 were all transient. All other IgG levels remained stable.

The final immunisation with S. minnesota R595 Re caused an early drop in IgG to LPS from E. coli R2, R3, 02, 06, 012, and 016, but levels to all but R3 recovered by the end of this study (day 156). Large increases to S. minnesota lipid A, S. typhimurium Ra, Rc and Re, P. aeruginosa PAC605, and E. coli R1 and K12 were obtained. Smaller, though significant, rises were obtained to E. coli J5, K12 lipid A, K12Re, 075, and 0111, K. aerogenes M10B, S. minnesota R-LPS, and to Rb and Rd LPS from S. typhimurium. Declining IgG

levels were obtained after initial increases to S. typhimurium Rb and Rd, P. aeruginosa PAC605, and E. coli J5, R1, R3, O2, and O6. Other LPS antigens showed no increase or decrease in IgG.

3:3:4. Rabbit 133.

The intention was to challenge this animal with a range of rough strains from E. coli. Unfortunately, this rabbit died after the third immunisation as antibody levels were beginning to show increases. As a result of the short course of this immunisation, results are not indicated.

3:3:5. Rabbit 134.

This received as immunogens a series of E. coli of O-serotypes predominant in cases of bacteraemia and septicaemia. IgG levels to most antigens before immunisation were negligible to low with the exception of S. typhimurium Re, E. coli R3, and E. coli K12, which all showed rising titres towards the first immunisation (figure 3:14a-g).

Challenge of rabbits with heat-killed E. coli O18 produced little or no response to most antigens, though moderate increases in IgG to E. coli O2, O75, R2, and R4, as well as S. typhimurium Ra and wild type LPS were detected.

E. coli O6 resulted in a massive immediate rise against the homologous LPS and to E. coli O16 and S. typhimurium Ra LPS. A large increase was also seen versus S. minnesota lipid A, but IgG returned to lower levels. IgG to S. typhimurium Rb, Rc, and Rd; S. minnesota Rb, Rc, Rd, and Re; P. aeruginosa Habs type 1; and E. coli R1, J5, K12Re, and O18 remained unaltered, and the remainder showed moderate

FIGURE 3:14a.
(Antigens and immunogens as described facing page 142.)

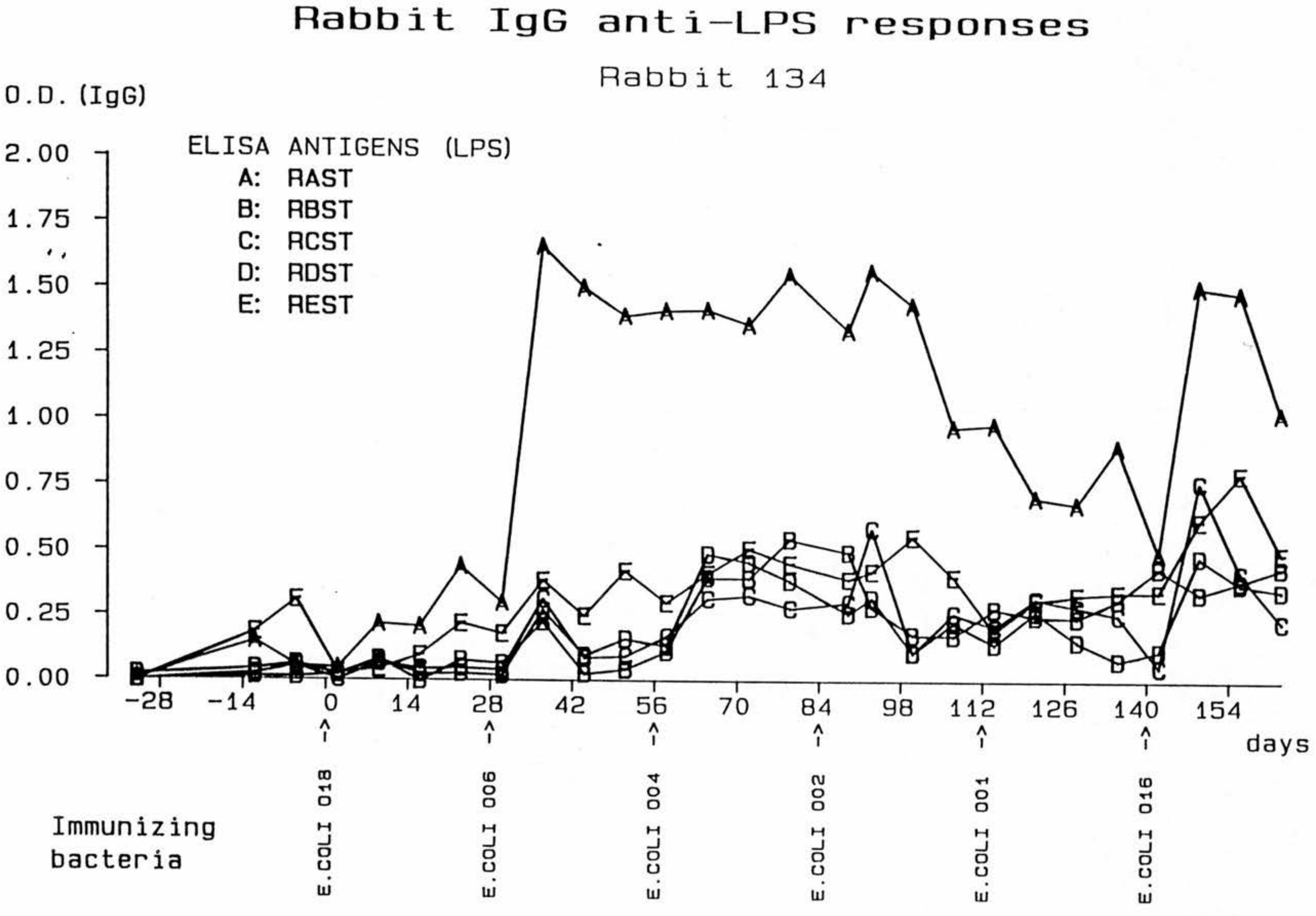


FIGURE 3:14b.
(Antigens and immunogens as described facing page 142.)

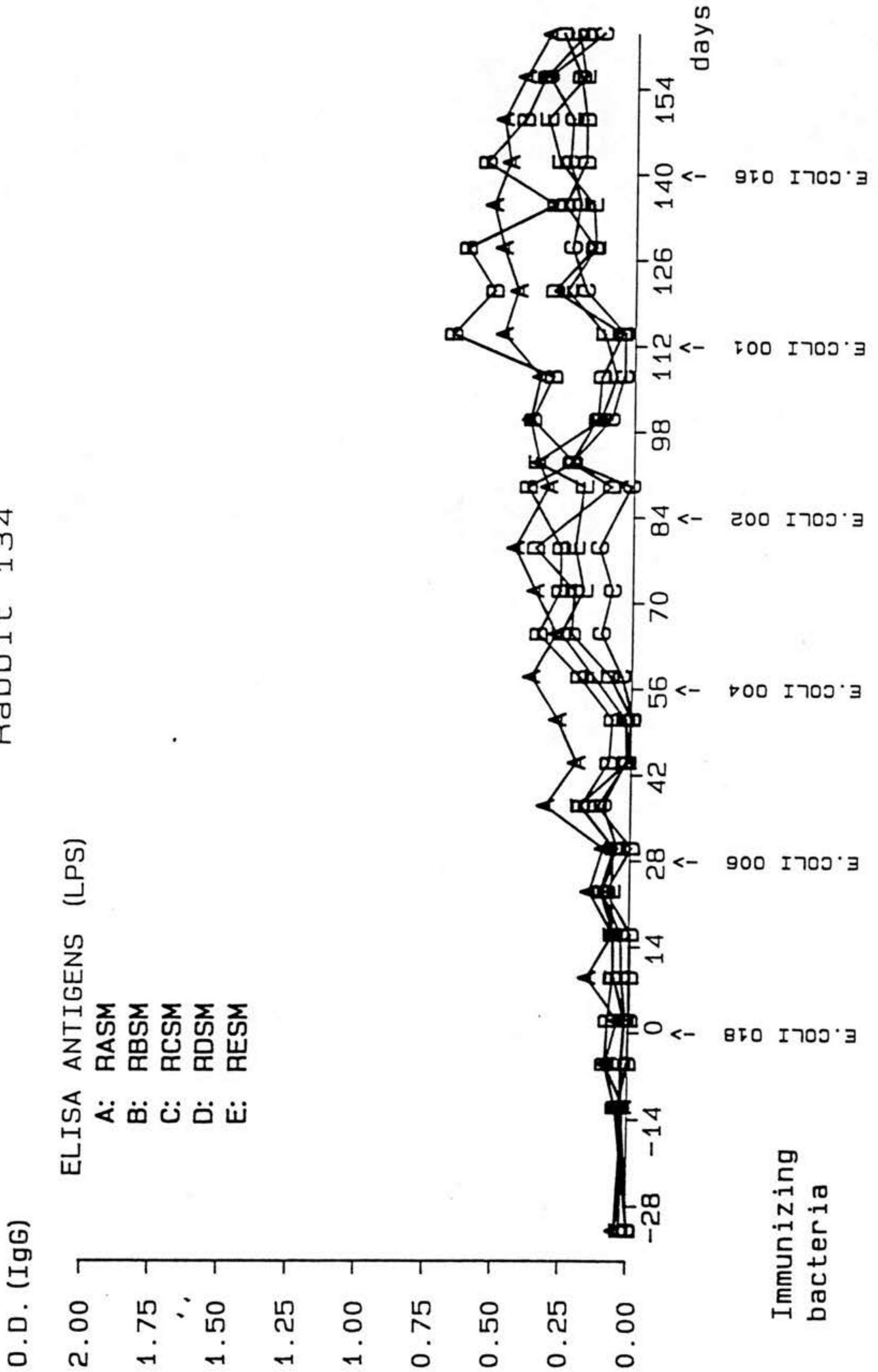


FIGURE 3:14c.
(Antigens and immunogens as described facing page 142.)

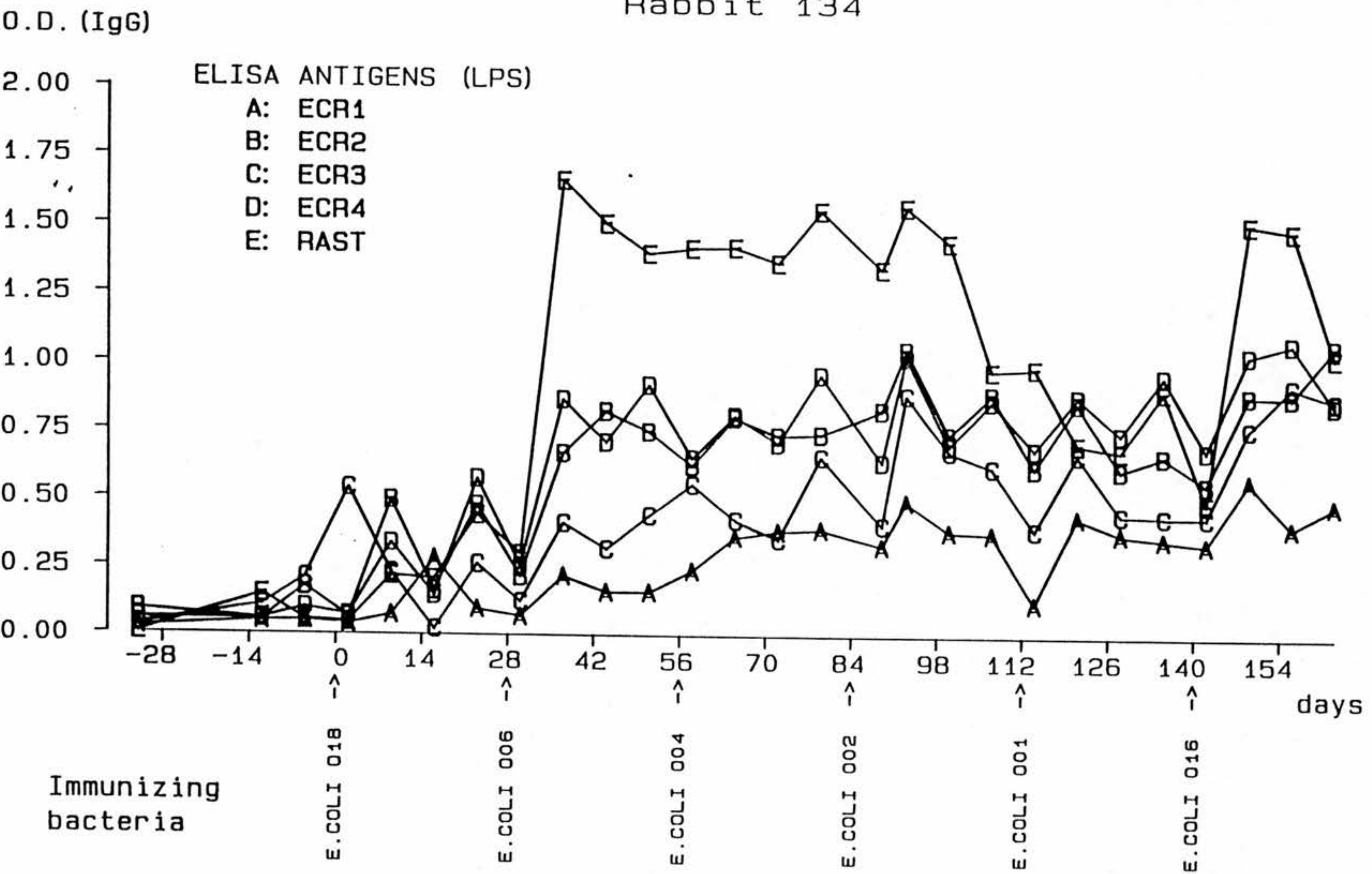


FIGURE 3:14d.
(Antigens and immunogens as described facing page 142.)

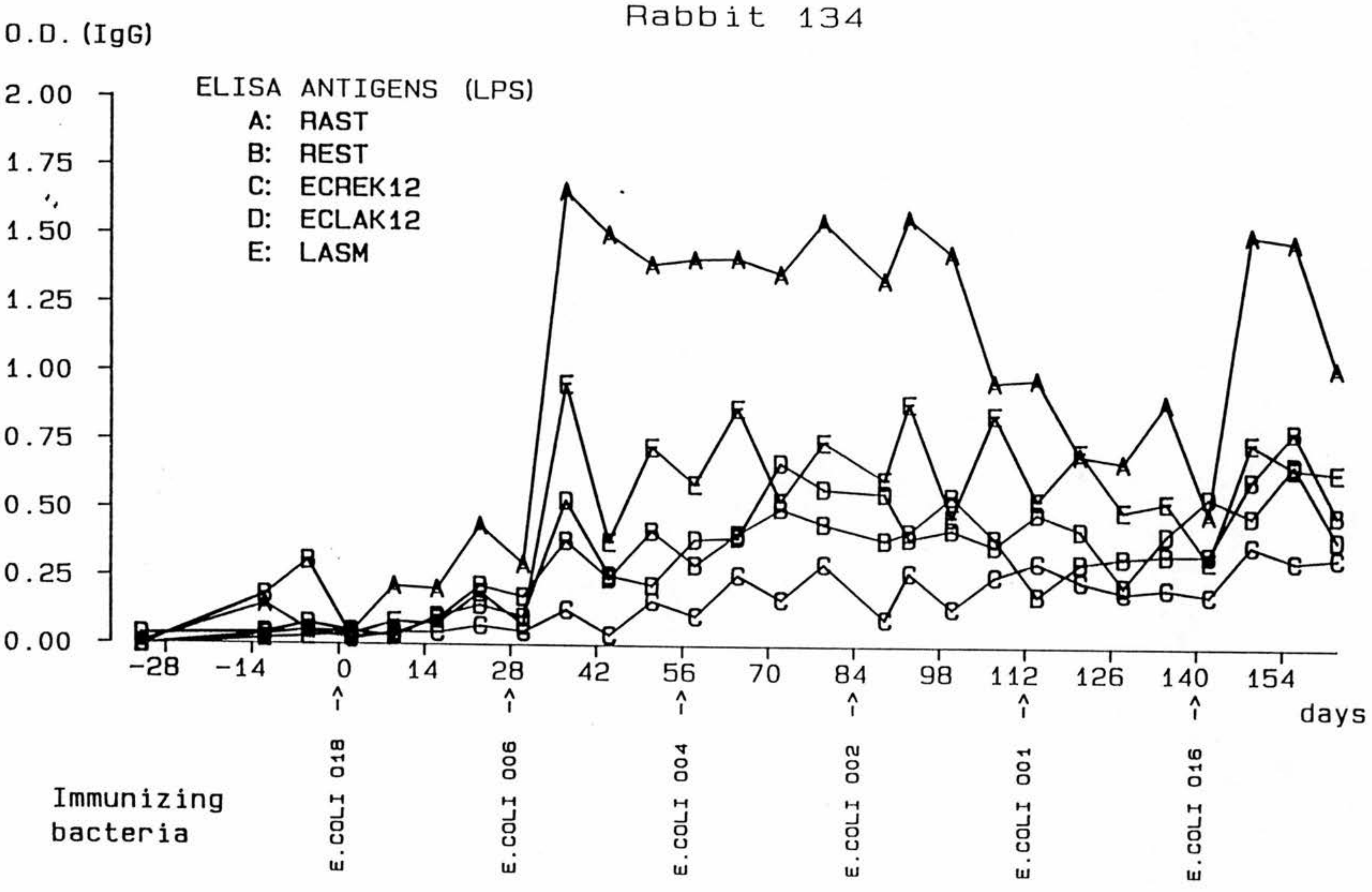


FIGURE 3:14e.
(Antigens and immunogens as described facing page 142.)

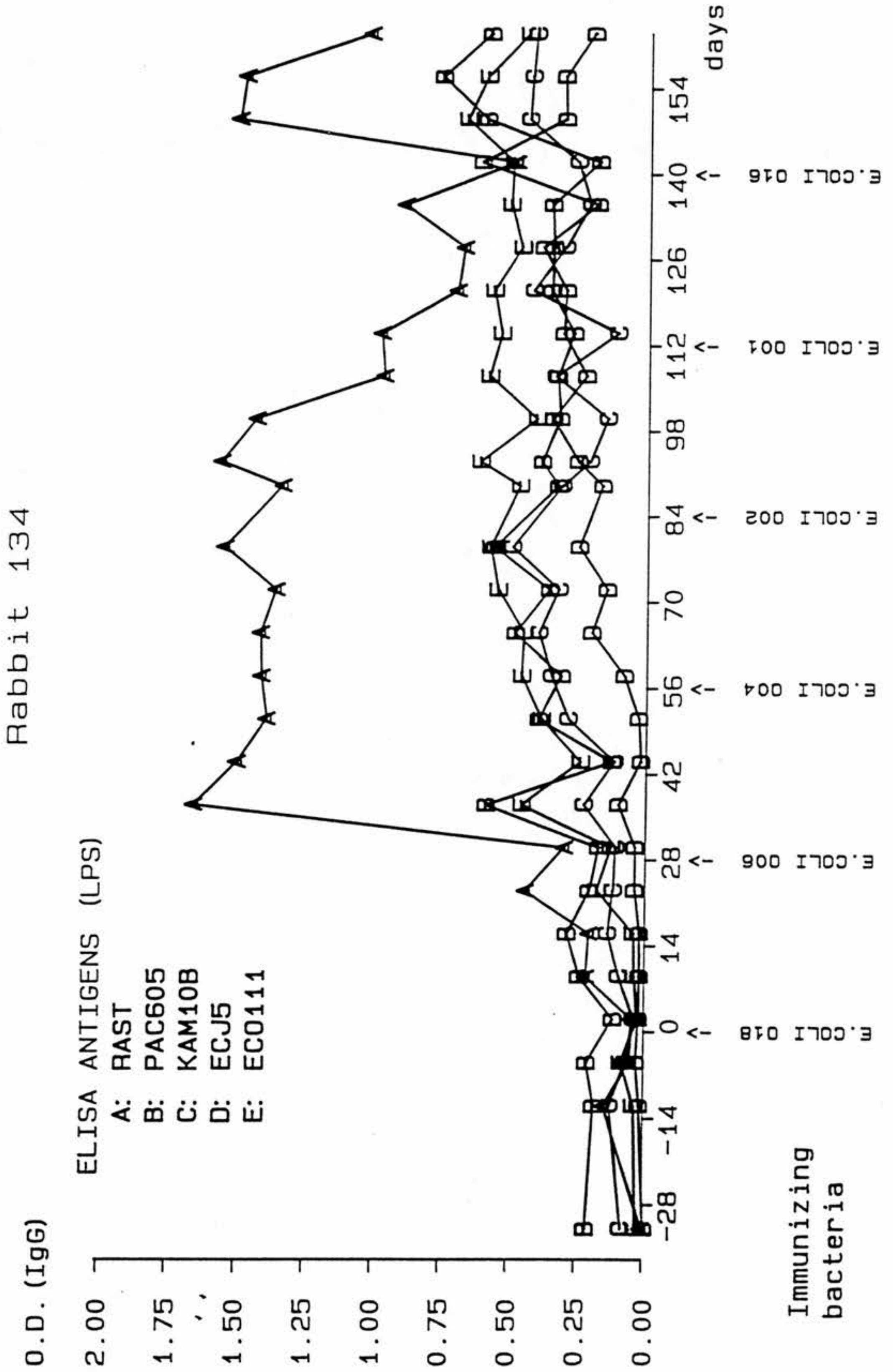


FIGURE 3:14f.
(Antigens and immunogens as described facing page 142.)

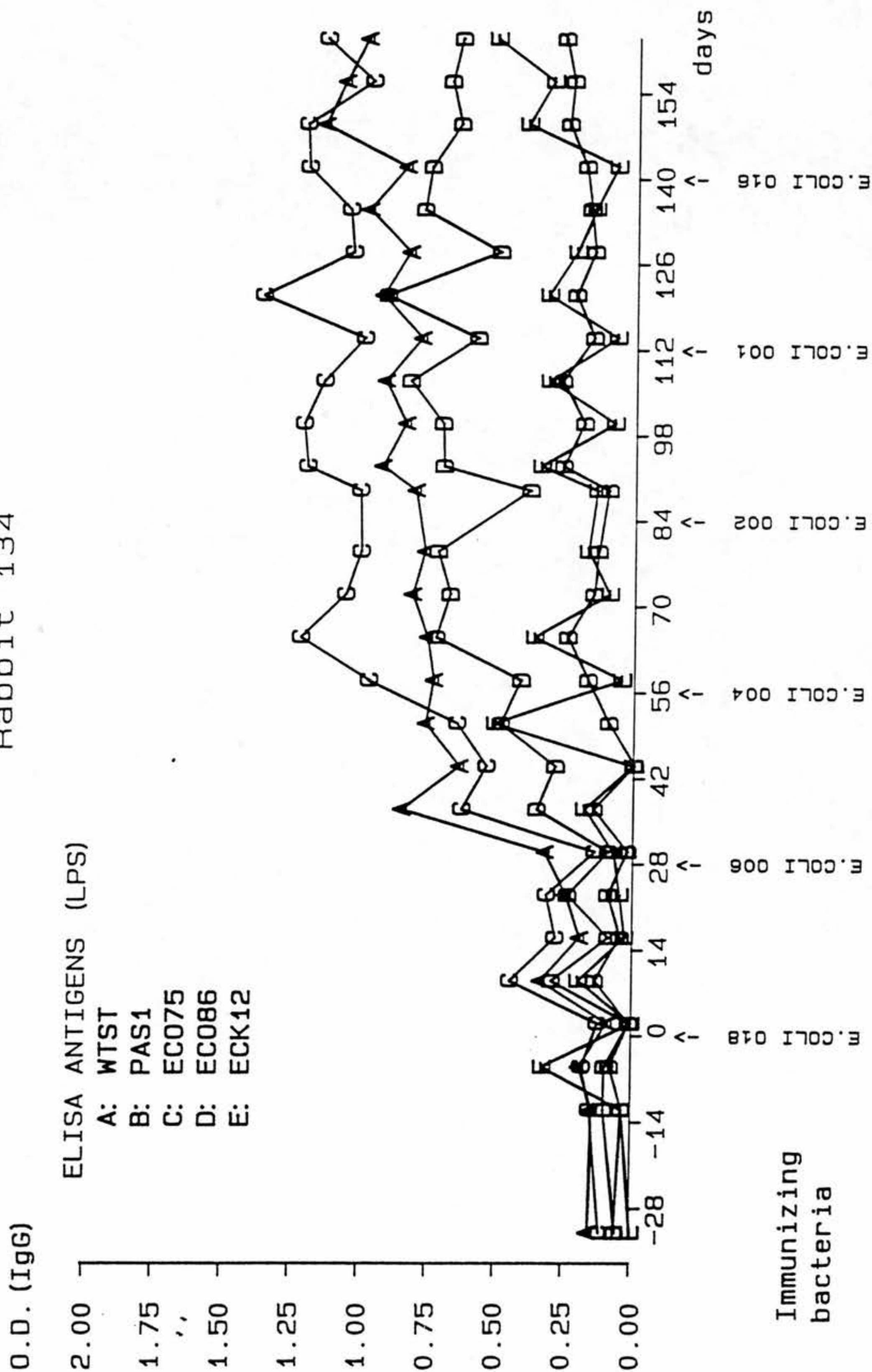
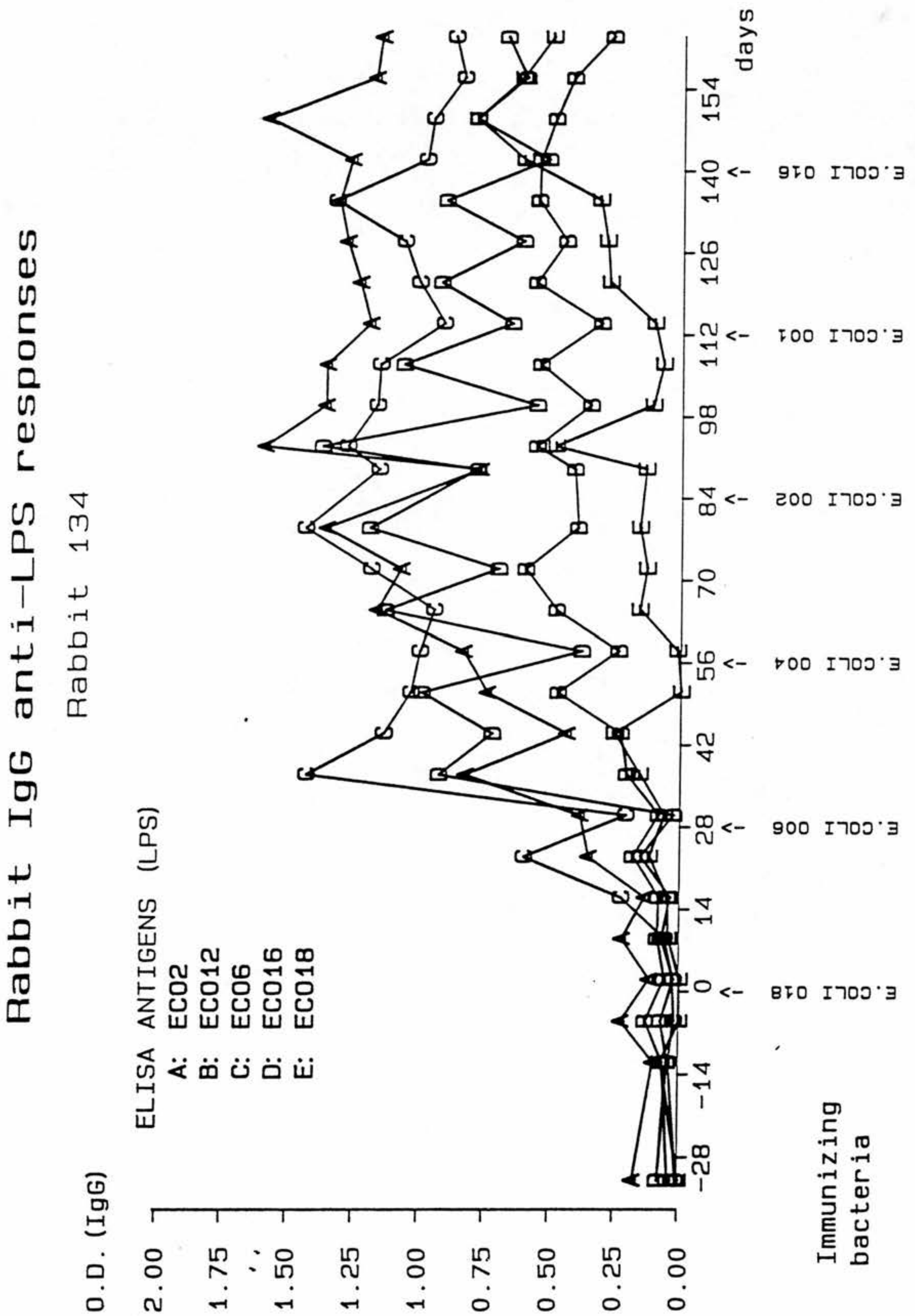


FIGURE 3:14g.
(Antigens and immunogens as described facing page 142.)



increases.

Inoculation with E. coli O4 produced little alteration versus most antigens, the exceptions being E. coli O16 (which showed large fluctuations within an underlying rising trend), O2 and O6 (which rose from high to very high levels), and O12 (which showed a transient rise to moderate levels.

Following immunisation with E. coli O2 a large increase was observed to homologous LPS from moderate to high levels, at which point IgG stabilised. This was paralleled by E. coli O18, but at very low levels. LPS from E. coli O16 again showed large fluctuations in IgG. Stability or small reductions were observed for many of the other antigens with the exception of S. typhimurium Rc, S. minnesota Rc, Rd, and Re, and E. coli R2, R3, and R4 which all showed falling titres following transient rises.

The next immunisation (E. coli O1) resulted in modest increases in IgG to all S. minnesota R-LPS and to E. coli O6, O18, and O12. Reduction in IgG occurred versus S. minnesota lipid A and S. typhimurium Ra, Rc, and Re. The remaining antigens showed relatively stable IgG reactivities.

Inoculation of the last immunogen (E. coli O16) produced a marked rise in only anti-S. typhimurium Ra IgG, although smaller increments were obtained versus several other LPS (S. typhimurium Rb, Rc, Rd, and Re; P. aeruginosa PAC605, S. minnesota lipid A; and E. coli R1, R2, R3, R4, K12, K12Re, K12 lipid A, O2, O16, and O18). Other S-LPS and R-LPS showed either little alteration or a modest fall in IgG.

3:3:6. Rabbit 135.

A range of O-antigen-containing organisms associated with

FIGURE 3:15a.
(Antigens and immunogens as described facing page 142.)

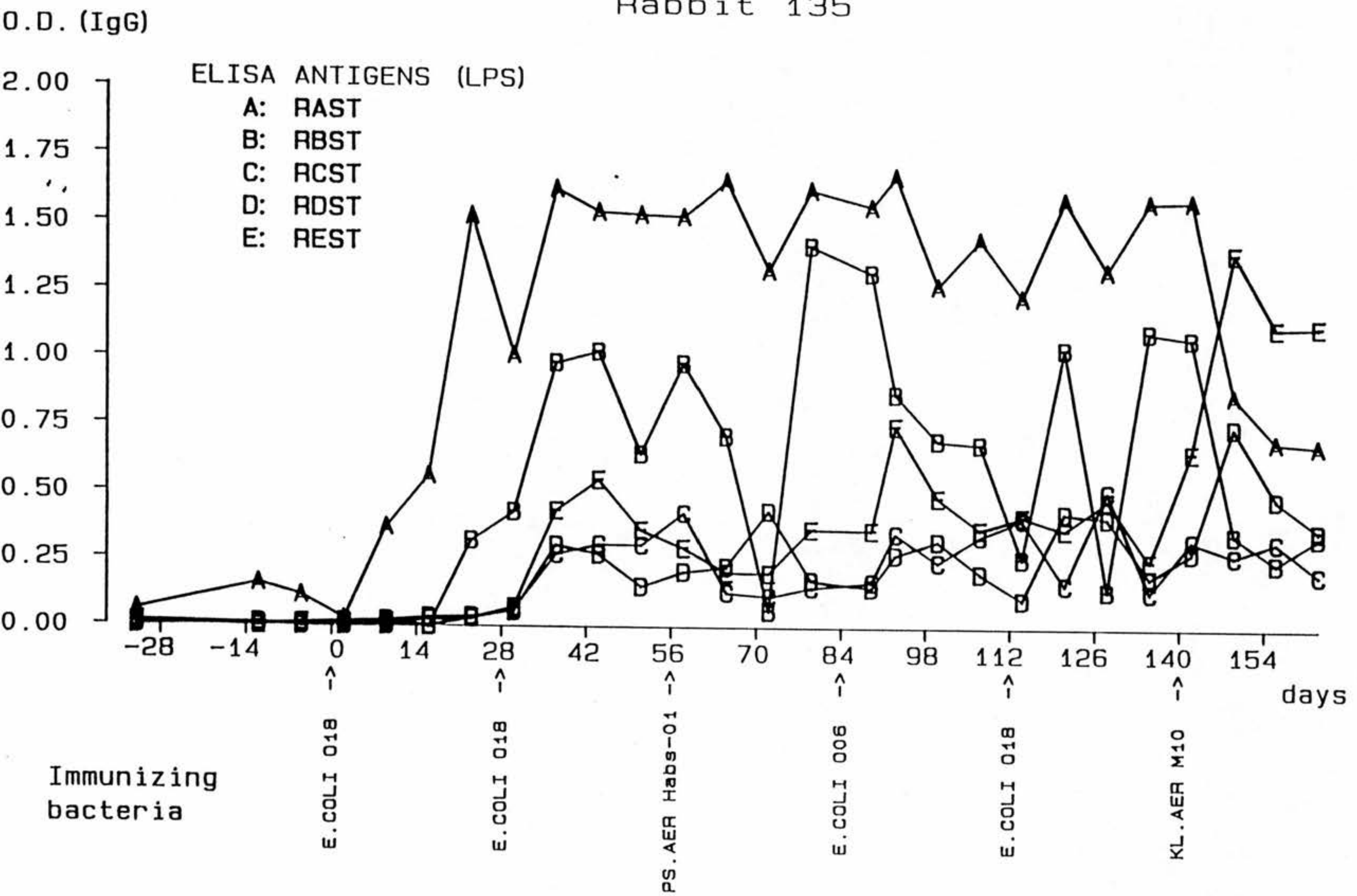


FIGURE 3:15b.
(Antigens and immunogens as described facing page 142.)

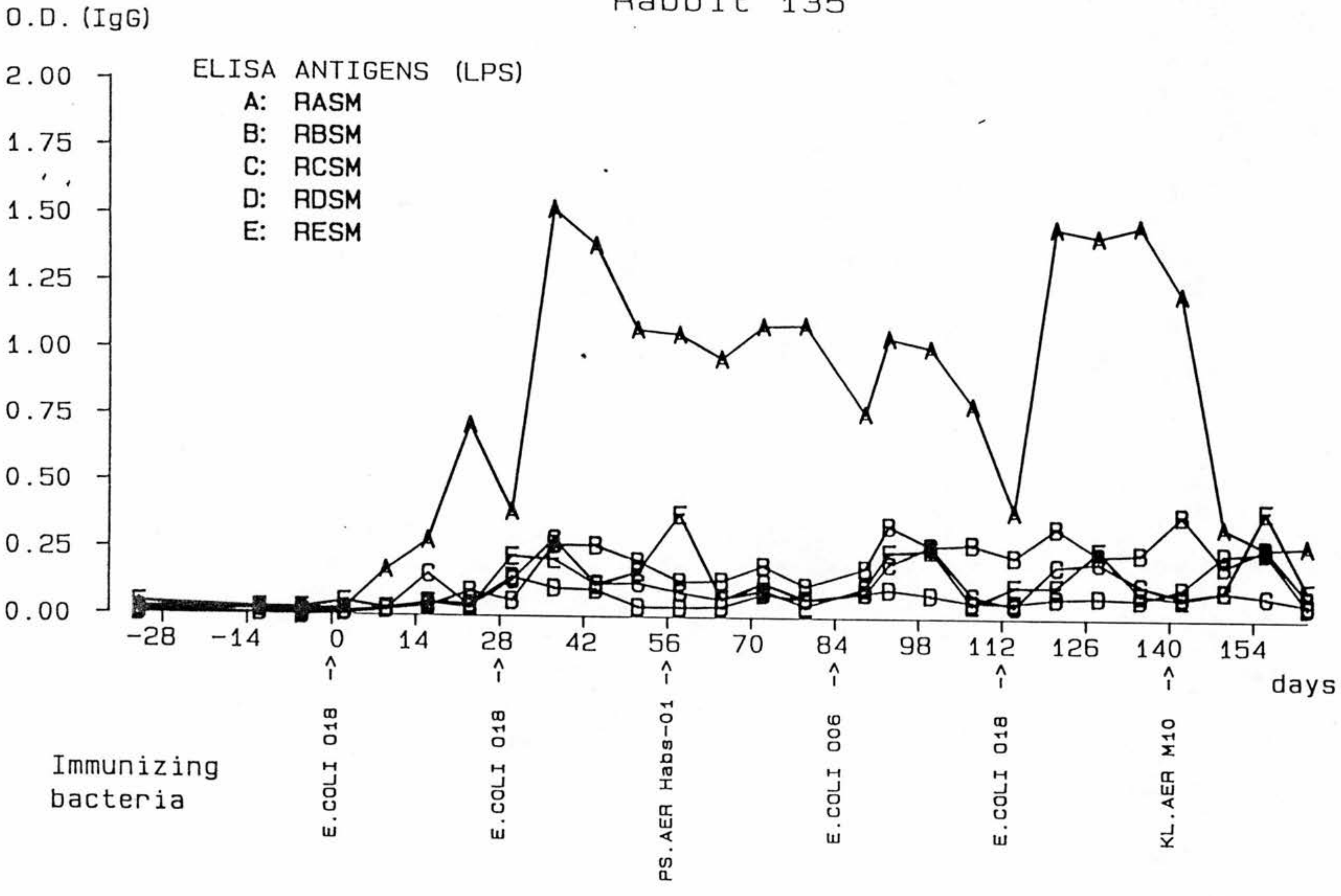


FIGURE 3:15c.
(Antigens and immunogens as described facing page 142.)

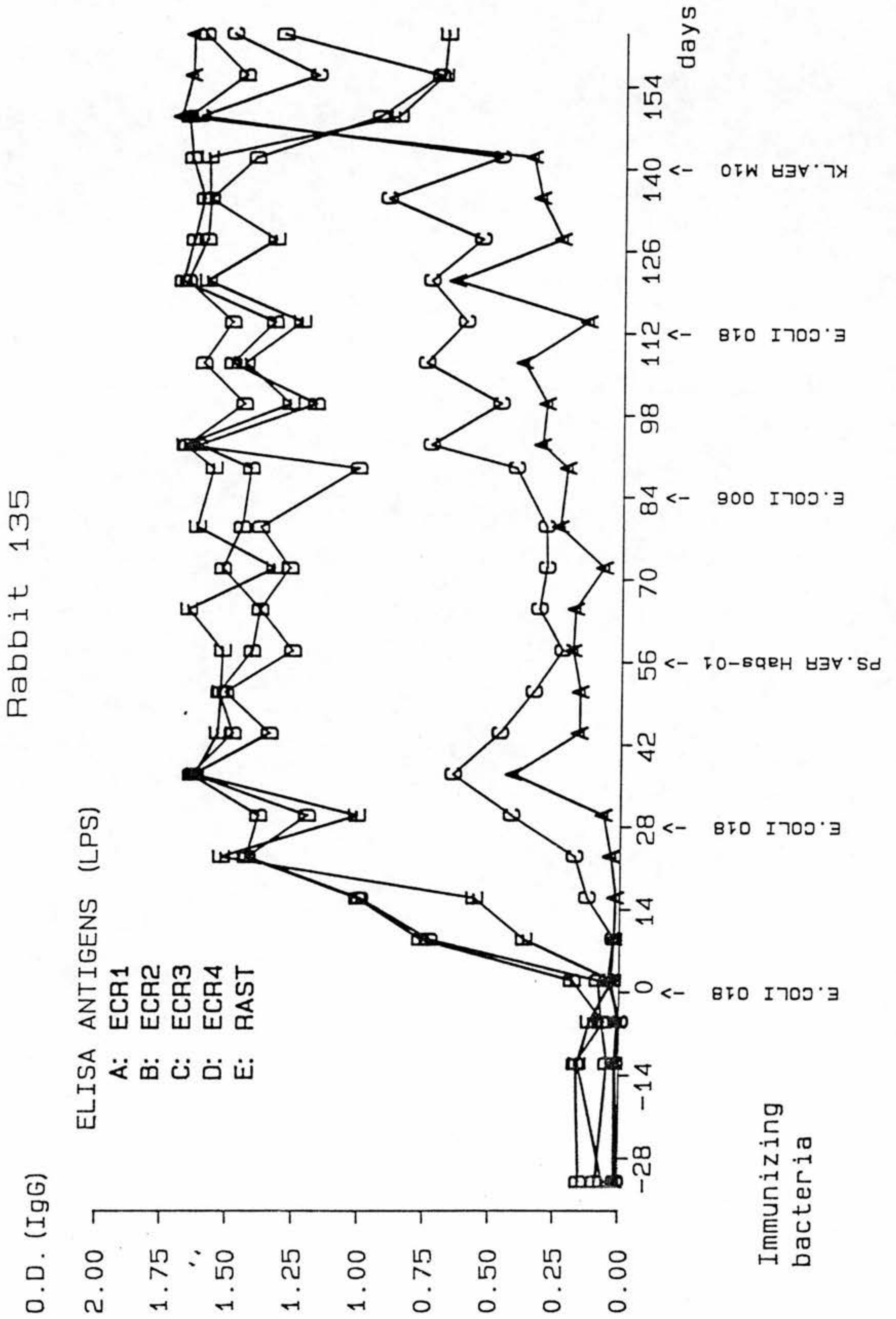


FIGURE 3:15d.
(Antigens and immunogens as described facing page 142.)

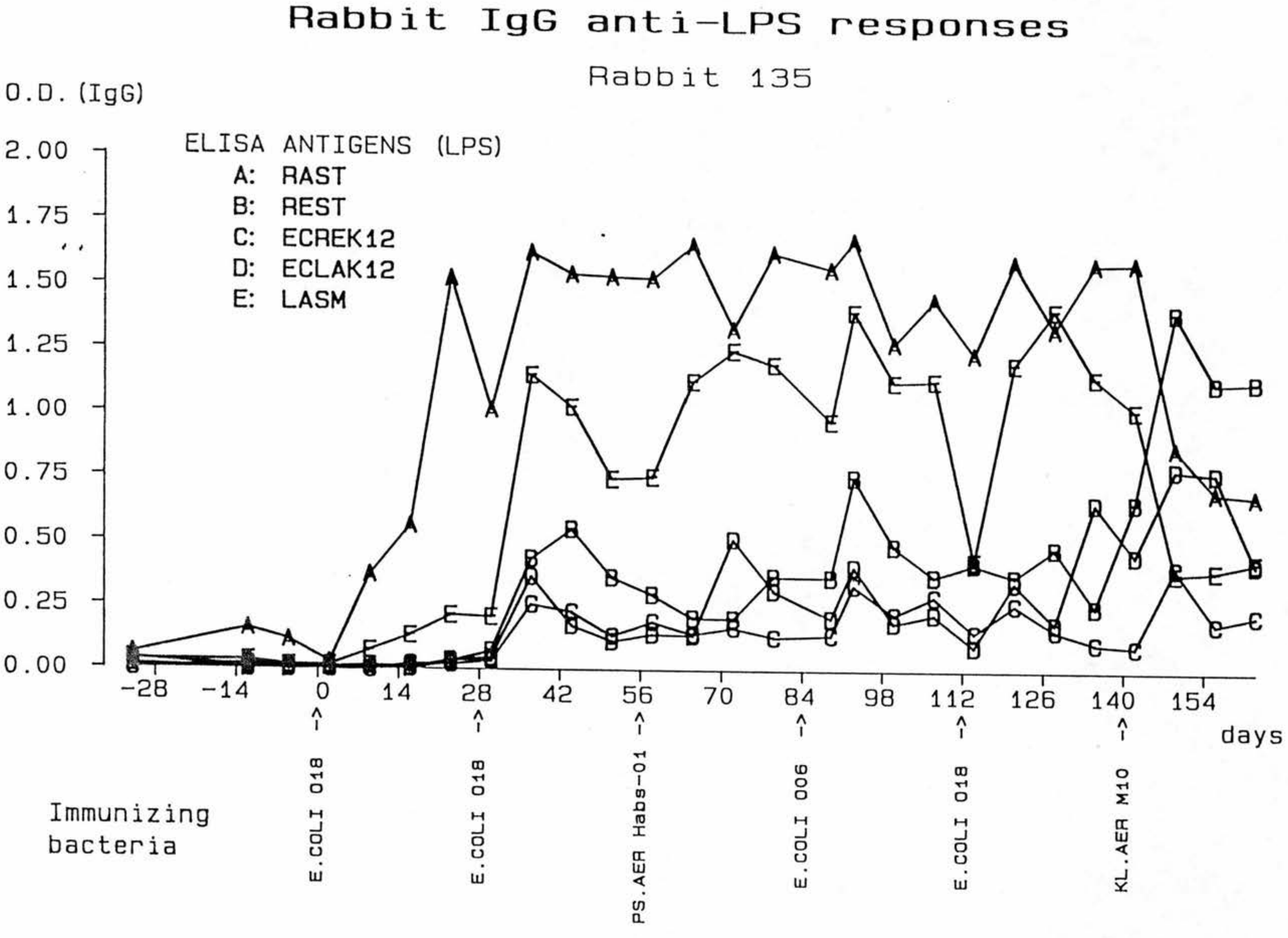


FIGURE 3:15e.
(Antigens and immunogens as described facing page 142.)

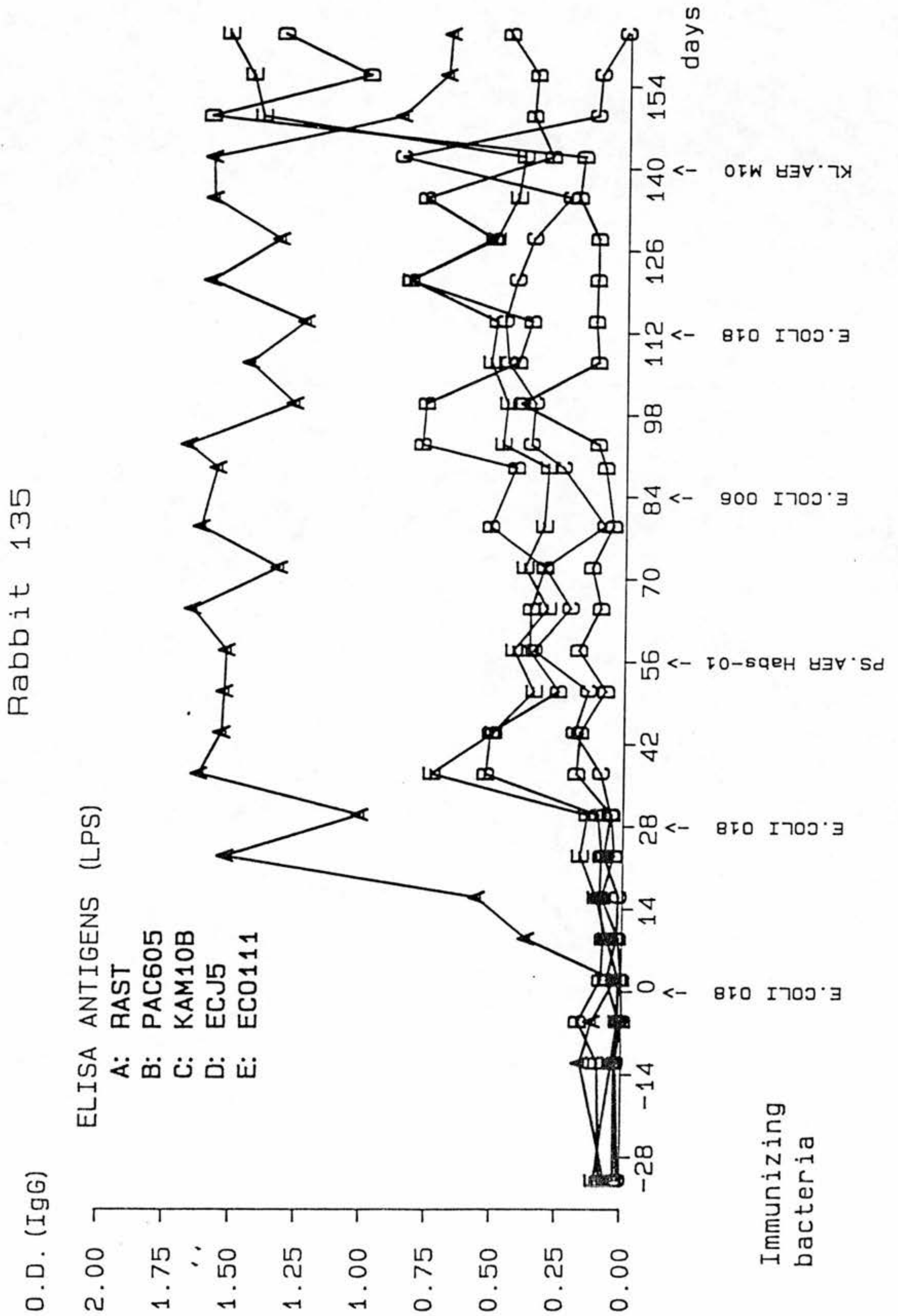


FIGURE 3:15f.
(Antigens and immunogens as described facing page 142.)

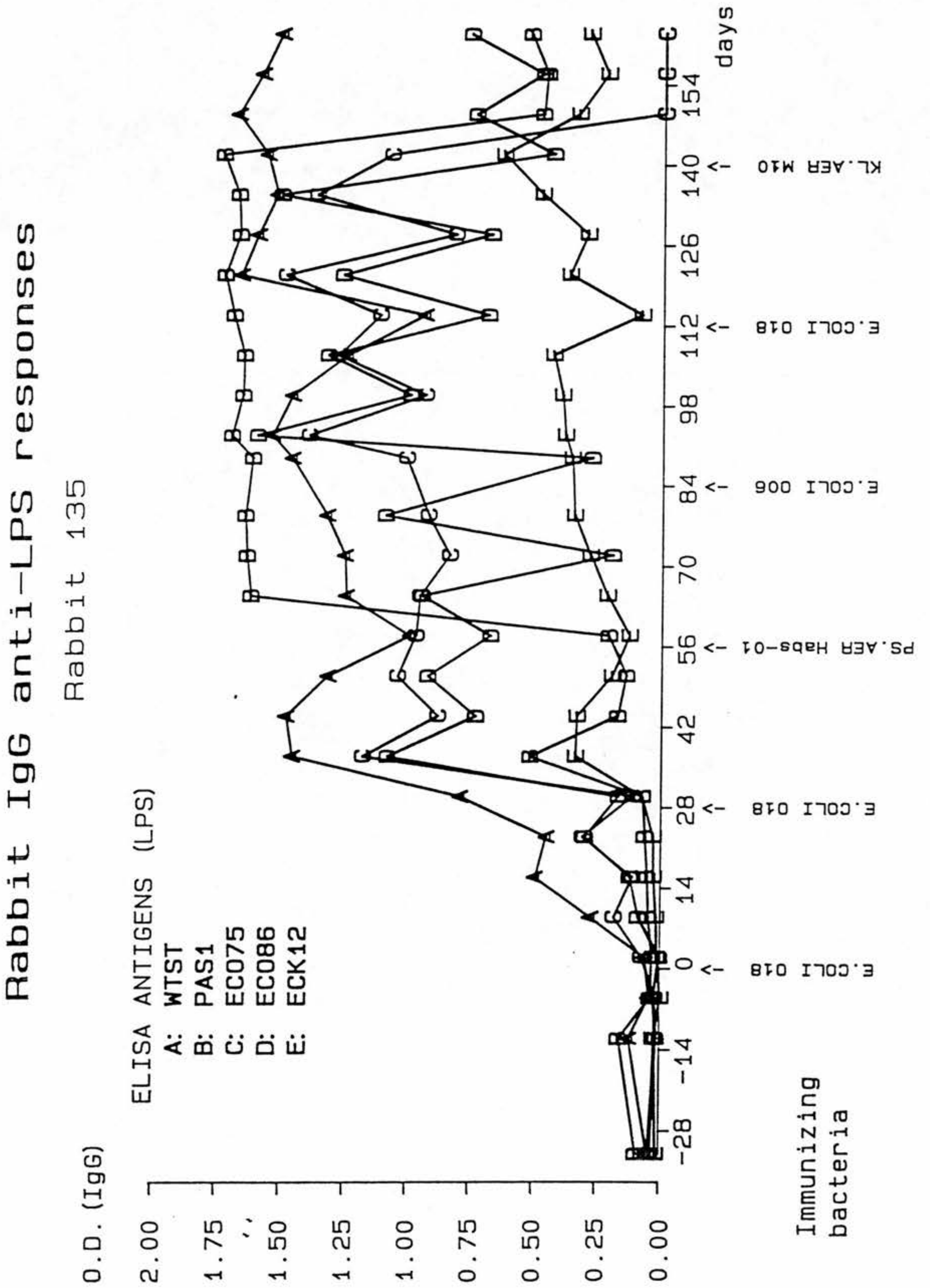
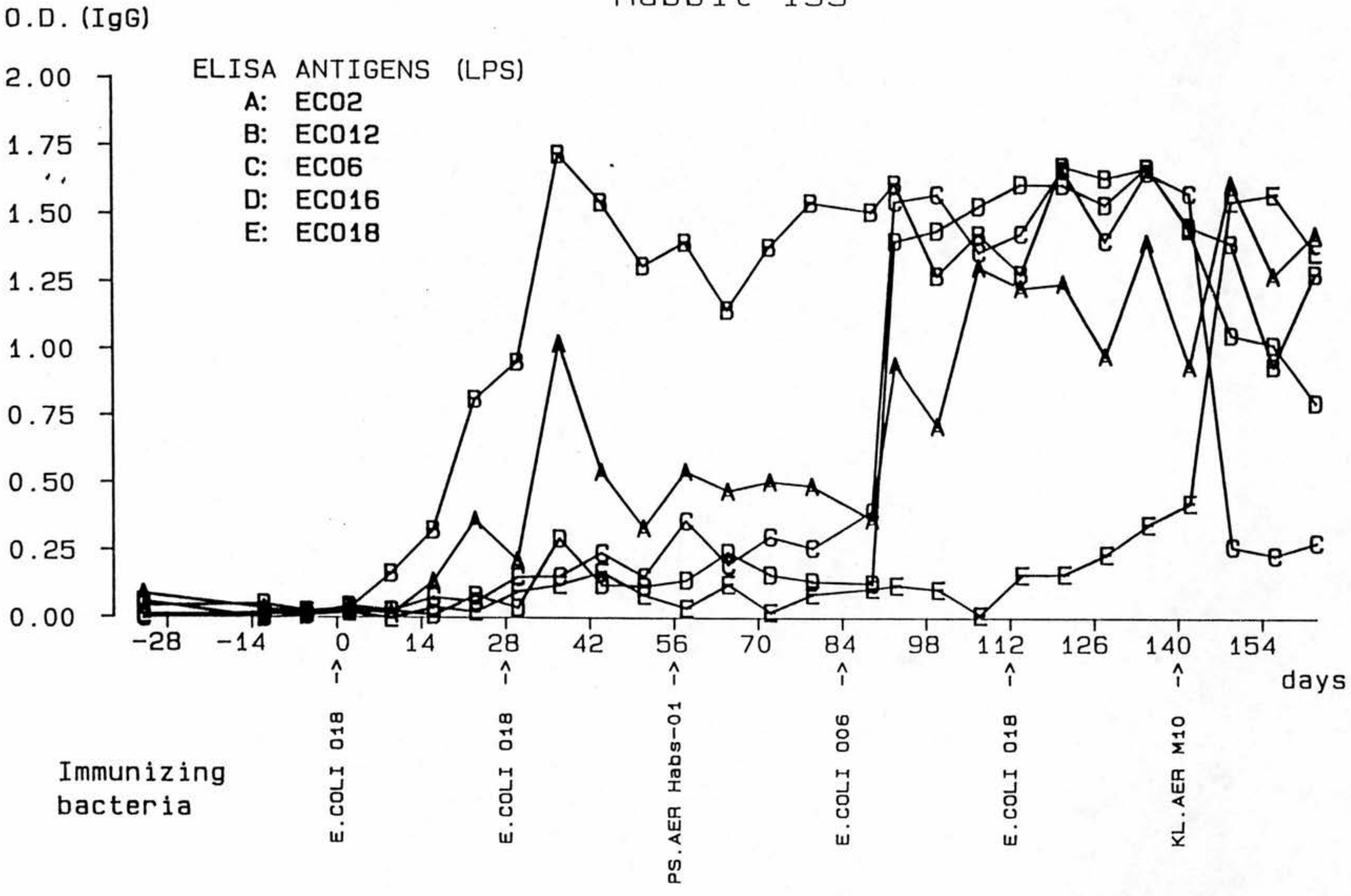


FIGURE 3:15g.
(Antigens and immunogens as described facing page 142.)



septicaemia from different genera were used to immunise this animal. Before the first immunisation (figure 3:15a-g), low levels of IgG were detected against *E. coli* R2 and R4, *P. aeruginosa* Habs type 1 and PAC605, and to *S. typhimurium* Ra and wild type.

Inoculation with *E. coli* O18 produced no significant homologous response, although moderate responses (*E. coli* O2, O75, and O86, and *S. typhimurium* wild type) and small responses (*E. coli* O6 and O111) to other antigens were obtained. Some rough antigens also showed increased recognition by IgG, with very steep rises observed to *S. typhimurium* Ra and *E. coli* R2 and R4 (which all showed detectable IgG before immunisation). A sharp rise was also observed versus *S. typhimurium* Rb, but this occurred 16 days after immunisation. Moderate rise were obtained to *E. coli* R3 and *S. minnesota* lipid A.

On subsequent immunisation with O18 cells, a very small transient rise was obtained to O18 LPS. Recognition of LPS from *E. coli* O12, O75, and O86 and *S. typhimurium* wild type were sharply and rapidly boosted to high levels though IgG then gradually fell in the period to the next immunisation. This immunisation also induced more modest rises in IgG recognising *E. coli* K12, O2 and O111, and *P. aeruginosa* Habs type 1, with IgG stabilising at moderate levels. *E. coli* O6 and O16 showed small but significant increases, and IgG to *E. coli* R2 and R4 and to *S. typhimurium* Ra were boosted to maximal levels, at which point they remained until the final immunisation. Significant and persistent increases were seen to *S. typhimurium* Rb and Rc, and non-persistent responses were seen to *S. typhimurium* Rd and Re, *S. minnesota* Ra and lipid A, and *E. coli* R1, R3, K12, K12Re, and K12 lipid A. Smaller increments were obtained versus most other antigens.

P. aeruginosa Habs type 1 produced a massive homologous response, with IgG remaining at maximal levels until the final immunisation. Lipid A from S. minnesota also showed IgG boosted to high levels, and transient increases were obtained against E. coli K12 lipid A, S. minnesota Re, and S. typhimurium Rb, Rc, and Rd. Small increments versus S. typhimurium wild type and E. coli 012 and 075 were obtained. The only other major change was observed with E. coli 086, against which massive fluctuations occurred.

E. coli 06 provoked a massive response to 06 LPS and to E. coli 016 and 086 LPS, although against 086, large fluctuations were present after the initial rise. A slower rise was observed versus E. coli 02, and a small response occurred to 0111. In contrast, IgG versus S. typhimurium wild type and E. coli 012 fell gradually. IgG to other S-LPS remained unaltered. Reduction in IgG levels also occurred for S. typhimurium Ra and Rb LPS. Little change was seen to S. typhimurium Rc and Rd, S. minnesota Ra and Rd, and E. coli R2, R4, K12, K12Re and K12 lipid A. Against other rough LPS, moderate increases were obtained, though these were not sustained.

The third immunisation with E. coli 018 initiated a moderate gradual rise to homologous LPS, and a modest rise wad obtained to 0111 LPS from this species. Once again lipopolysaccharide from E. coli 075 and 086 showed massive fluctuations in IgG and other IgG to smooth LPS remained relatively stable. After an initial sharp reduction at immunisation, anti-S. minnesota lipid A antibodies returned to previous levels. Boosted responses occurred to LPS from S. typhimurium Rb and S. minnesota Ra, but the former response showed large fluctuations. Smaller rises were obtained to P. aeruginosa PAC605, S. typhimurium Rd, and E. coli R1, R3, and K12 lipid A. All

other LPS showed unaltered IgG levels.

The final immunisation with *K. aerogenes* M10 resulted in a moderate but transient rise versus its O-antigen deficient mutant M10B. Moderate increases were also obtained versus *E. coli* O2 and *S. typhimurium* Rd, with larger, more sustained increases against *E. coli* R1, R3, J5, O18 and O111, and *S. typhimurium* Re apparent. In contrast to most immunogens, this one caused large reductions in IgG to *P. aeruginosa* Habs type 1, *S. minnesota* Ra and lipid A, *S. typhimurium* Ra, and *E. coli* R4, O6, O16, O75 and O86, and also more moderate reductions to *P. aeruginosa* PAC605 and *E. coli* K12 and O12 were also observed. Only anti-R4 and anti-O12 IgG showed signs of recovery from these reductions. IgG to other antigens remained unaltered from previous levels.

3:4. Antigenic Presentation of LPS-polymyxin in ELISA.

3:4:1. Assay of 4 Normal Human Sera in ELISA.

ELISA was carried out on four normal human sera (NHS) selected for high (GL+), medium (MED1 and MED2), and low (GL-) levels of IgG in the previously described CGL-pool assay. ELISA strips were prepared as described in MATERIALS AND METHODS with a variety of preparations from S. typhimurium R878 (Rc chemotype). The preparations used were: i) purified LPS, ii) LPS-polymyxin complex, iii) outer membrane - OM - extracts, and iv) heat-killed bacteria. All antigens were coated onto plates with approximately equivalent concentrations of carbohydrate, and a control was prepared which was coated with post-coat only. Each NHS was prepared in a doubling dilution series from 1:25 to 1:1600 plus a non-antibody control for assay against each antigen. Results were obtained as absorbance at 590nm, which gave an indication of levels of IgG.

The ELISA results are presented in figures 3:16a-d. For each of the four NHS, blank strips produced negligible absorbances at all dilutions, as did control wells for each antigen with no serum. The one exception to this was the heat-killed cells with MED1 but, as all other heat-killed cells controls possessed only minimal absorbance, this was probably due to a stray contaminant.

By comparing the profiles of each NHS in figure 3:16, it can be seen that GL+ possessed the highest overall levels of IgG, MED1 and MED2 both possessed more moderate IgG levels, and GL- possessed the lowest. The absorbances obtained against LPS-polymyxin at a dilution of 1:100 (the dilution used in the assay) were 0.191 (100%) for GL+, 0.046 (24%) for GL-, and 0.062 (32%) and 0.212 (111%) for MED1 and

Figure 3:16a

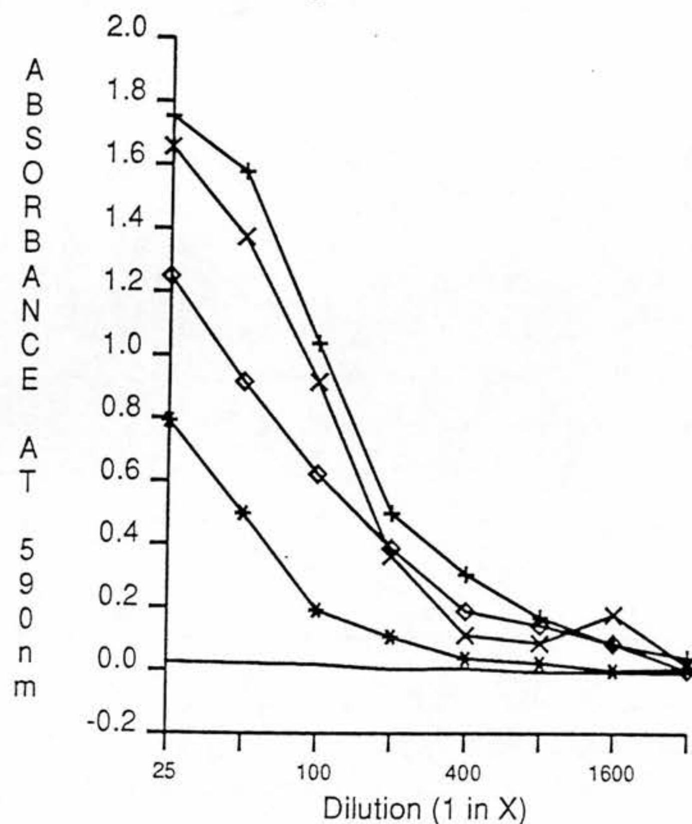


Figure 3:16b

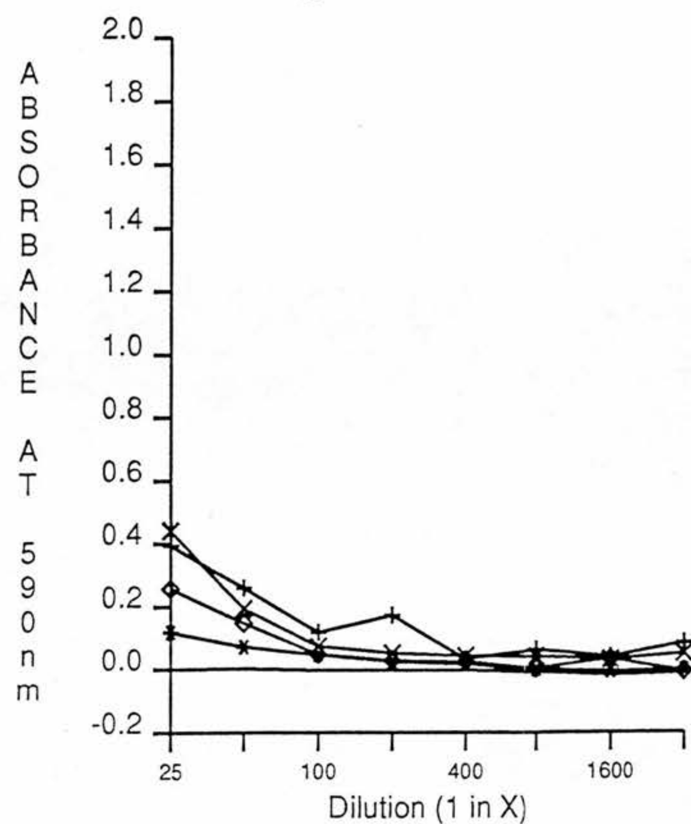


Figure 3:16c

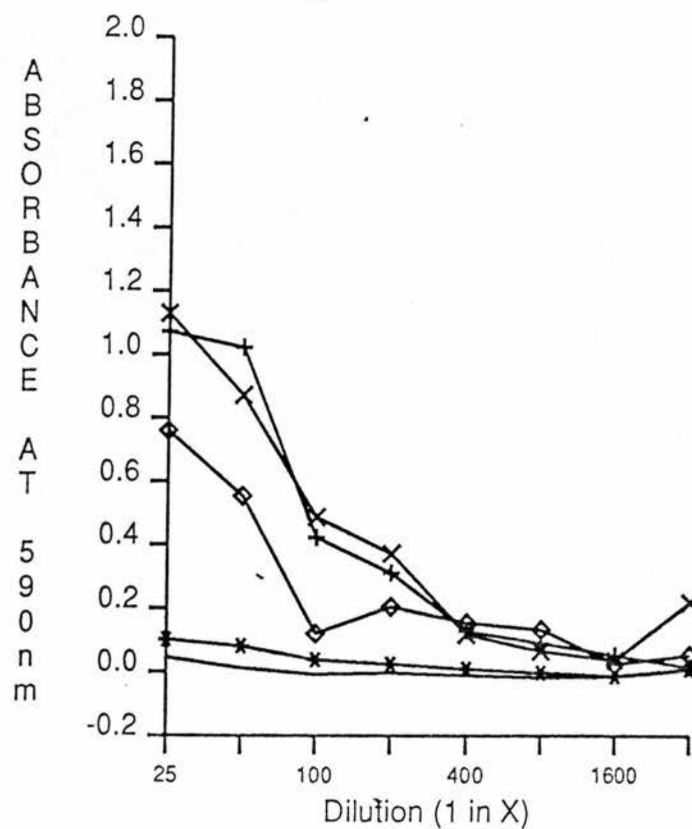


Figure 3:16d

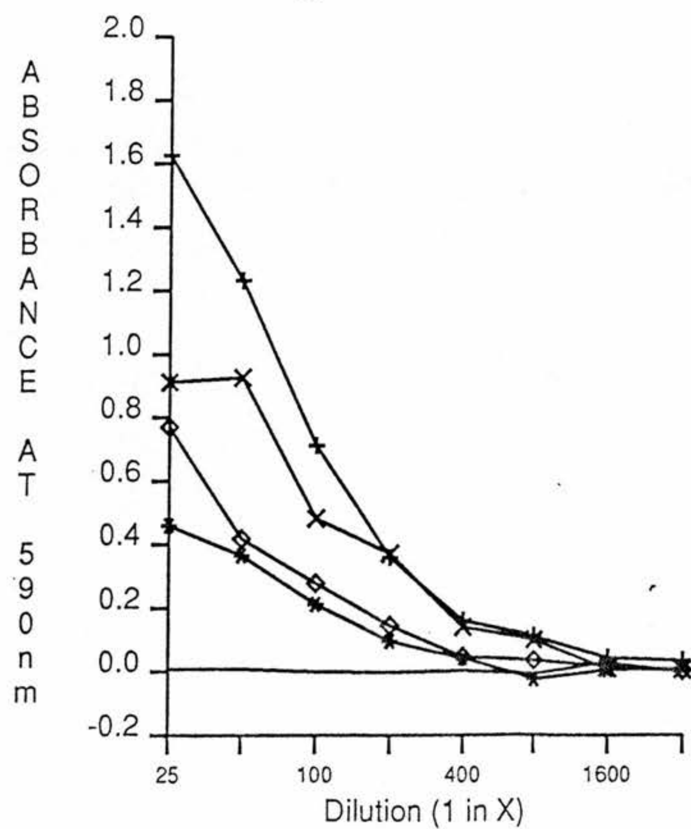


FIGURE 3:16. Binding activities of four human sera against four LPS-containing antigens (as described in the legend) from S. typhimurium R878 and a blank in ELISA. A doubling dilution series of serum from 1:25 to 1:1600 plus a control containing no serum was used. Figures (a), (b), (c), and (d) represent the activities of GL+, GL-, MED1, and MED2 sera respectively.

MED2 respectively. The results reflected reasonably closely those obtained for these four NHS in previous assays: GL+ 100%; GL- <10%; MED1 approximately 60%; and MED2 approximately 110%.

Of the four antigens, purified LPS and OM produced the highest absorbances with all sera, both antigens producing very similar absorbances at all points. Heat-killed bacteria represented the next most reactive antigen with LPS-polymyxin producing the lowest absorbance values.

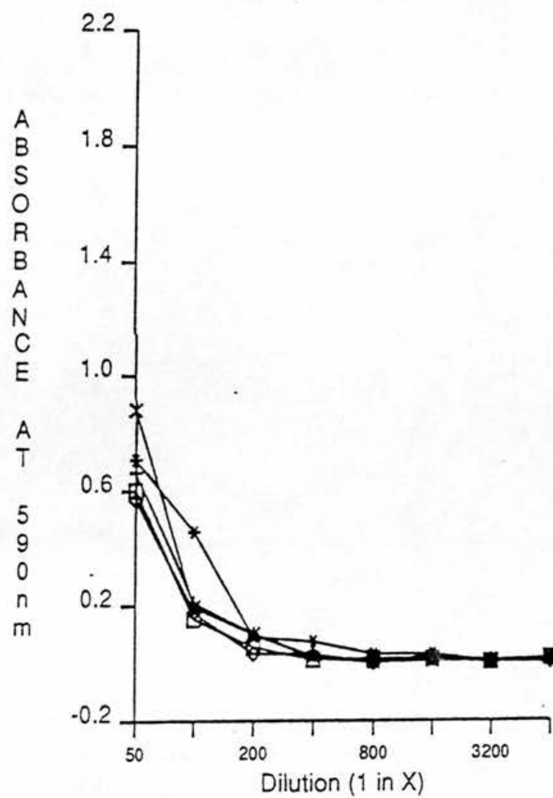
3:4:2. Effect of Absorption with Bacteria on IgG Levels to LPS.

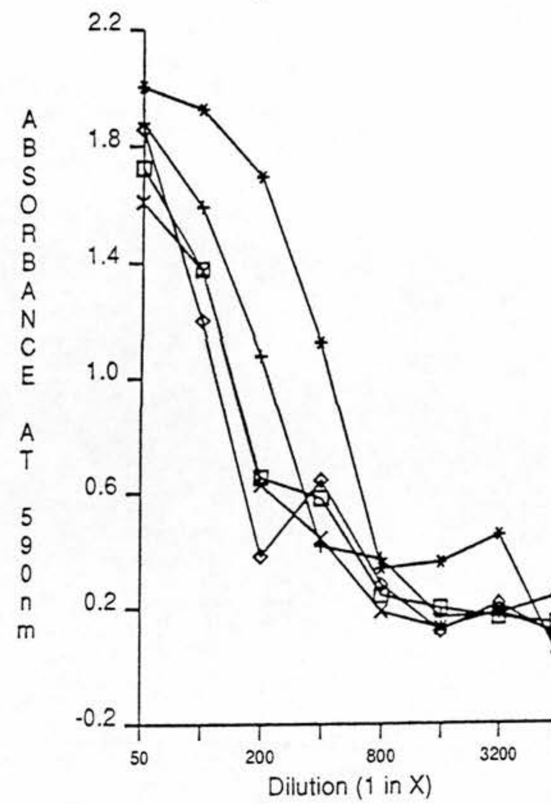
A series of absorptions was carried out on each of the 4 NHS with *S. typhimurium* R878 bacteria. NHS and heat-inactivated (56°C for 30min) HNHS were absorbed with heat-killed and viable organisms respectively. Heat-killed bacteria were used to absorb untreated serum as complement is strongly bacteriolytic for organisms with R-LPS on their surface, and viable organisms can be used to absorb heat-inactivated serum as the complement activity is removed by heating.

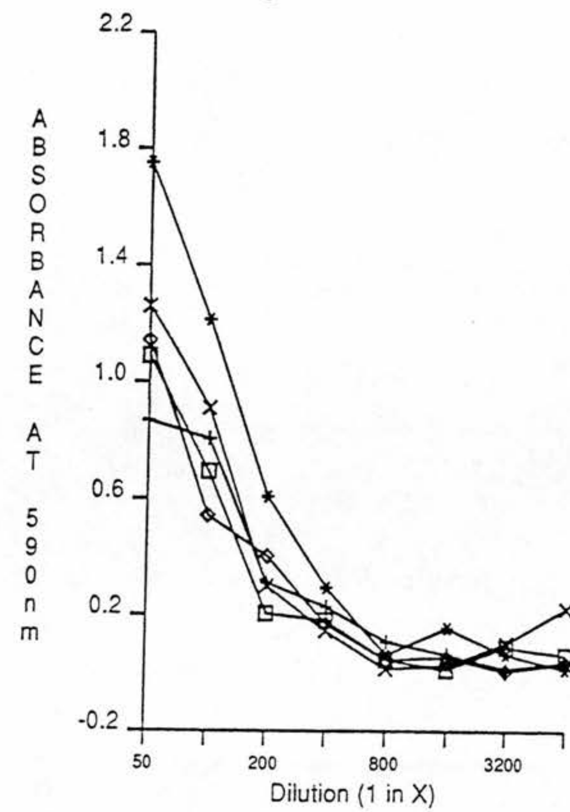
a) Absorption of NHS with heat-killed bacterial cells.

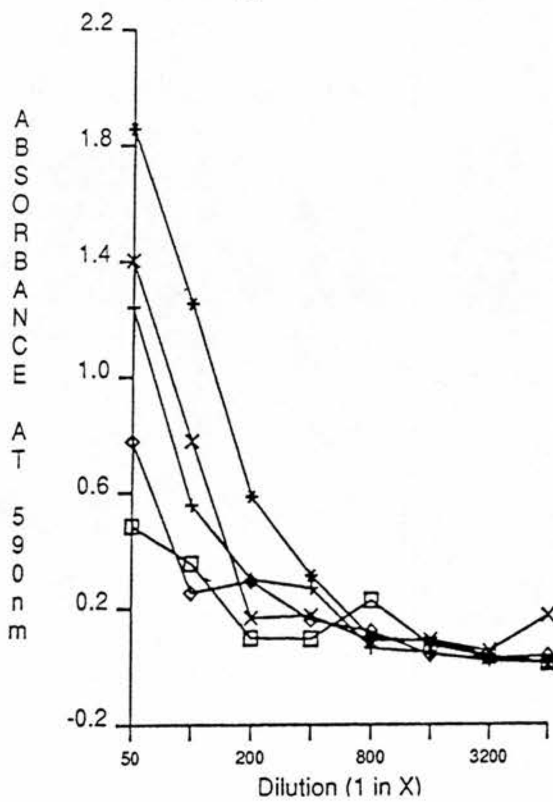
The results obtained by absorption of 4 NHS with heat-killed bacteria were graphed (figures 3:17, 3:18, 3:19, and 3:20). Four solid-phase antigens (LPS-polymyxin, LPS, OM, and heat-killed bacteria) from *S. typhimurium* R878 in addition to a control were used for detection of IgG in ELISA.

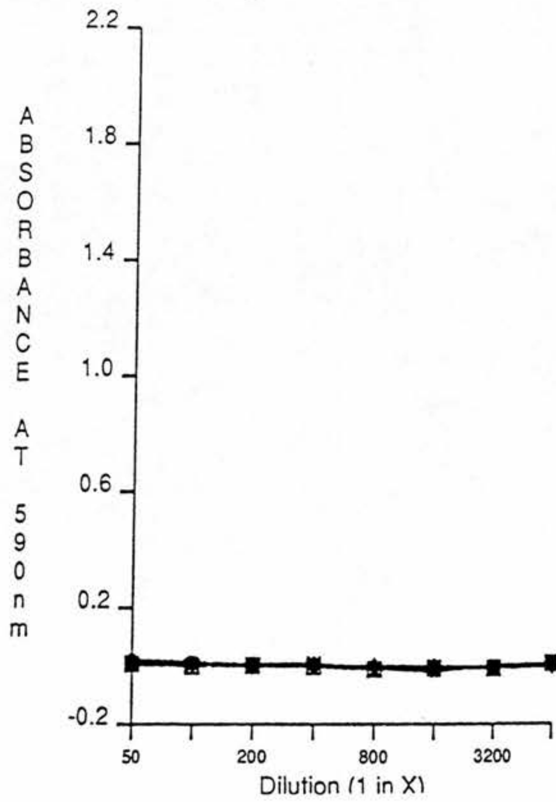
The results with unabsorbed sera were similar to those achieved previously (see figure 3:16). Four cycles of absorption were carried out for each NHS, and the results are presented for each. IgG in each serum can be seen to fall after each absorption step. Little











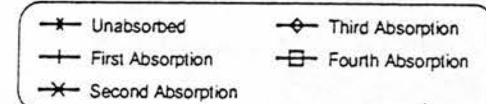
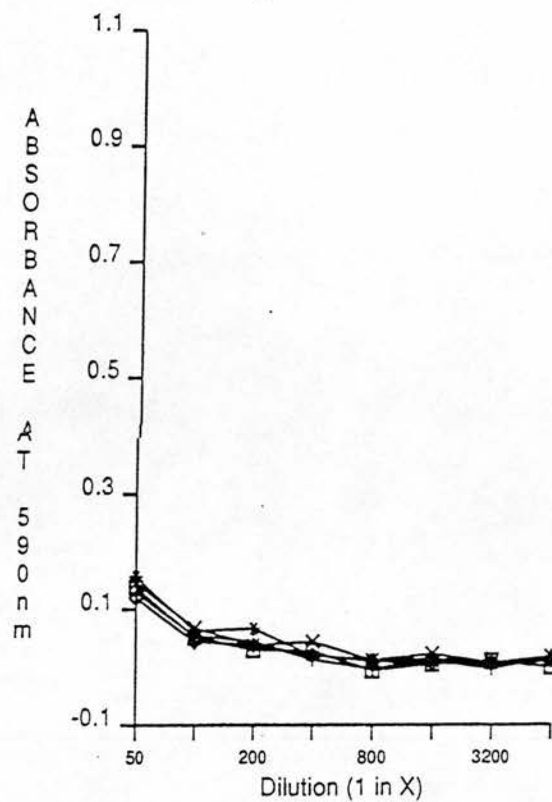
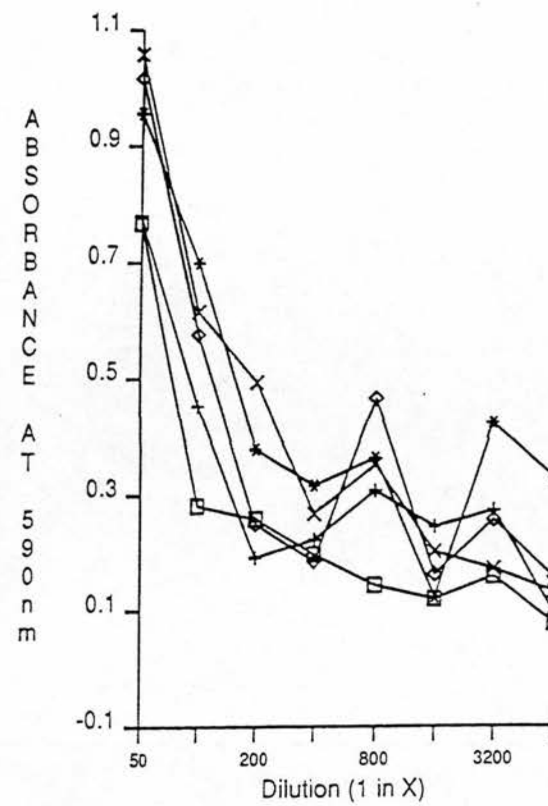
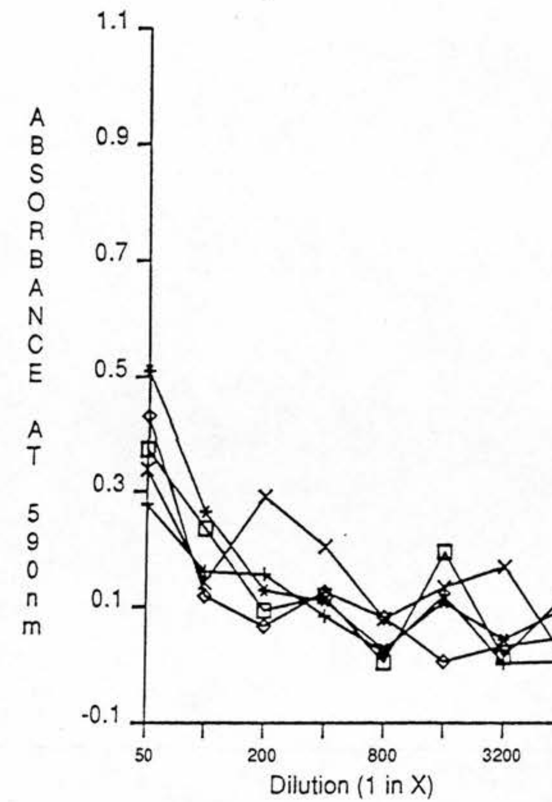
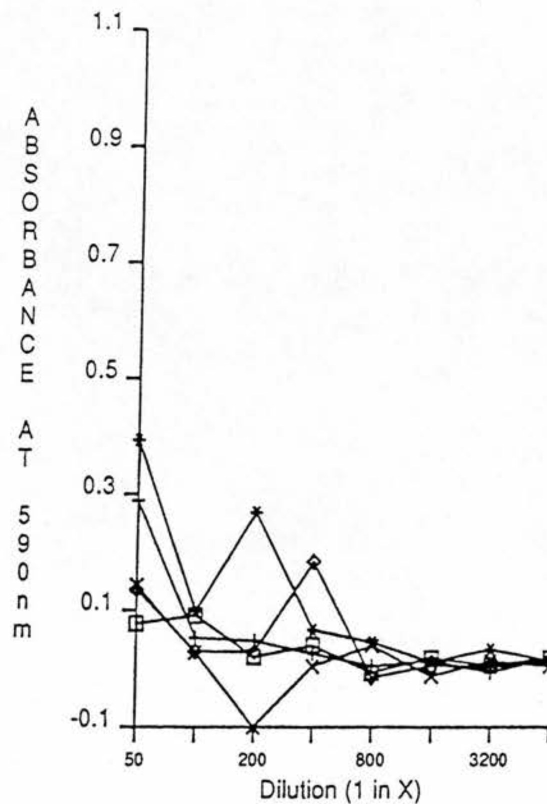


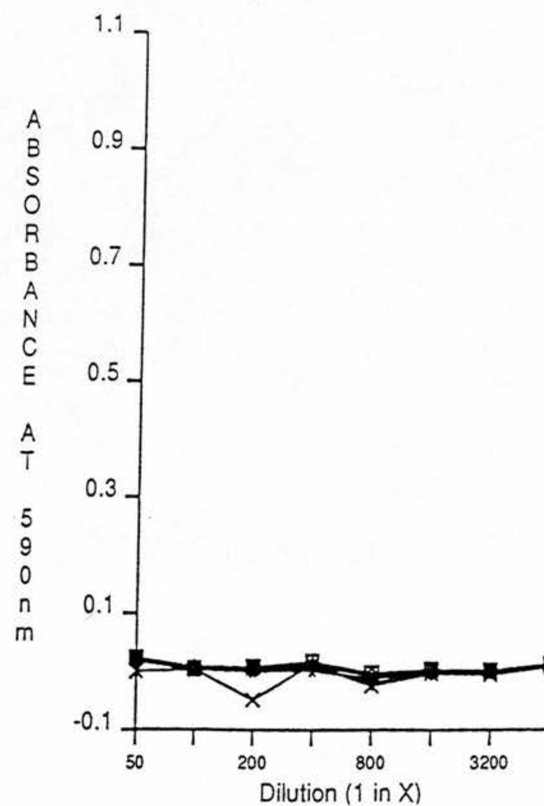
FIGURE 3:17. Binding activities of GL+ serum against five antigens in ELISA before absorption and after four consecutive absorptions with heat-killed _S. typhimurium_ R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.











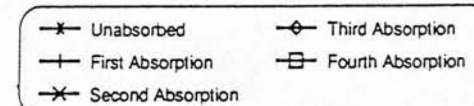
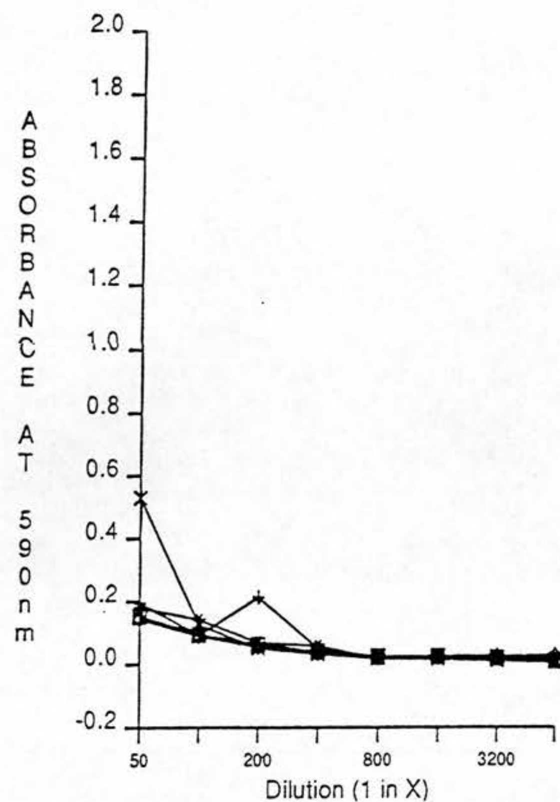
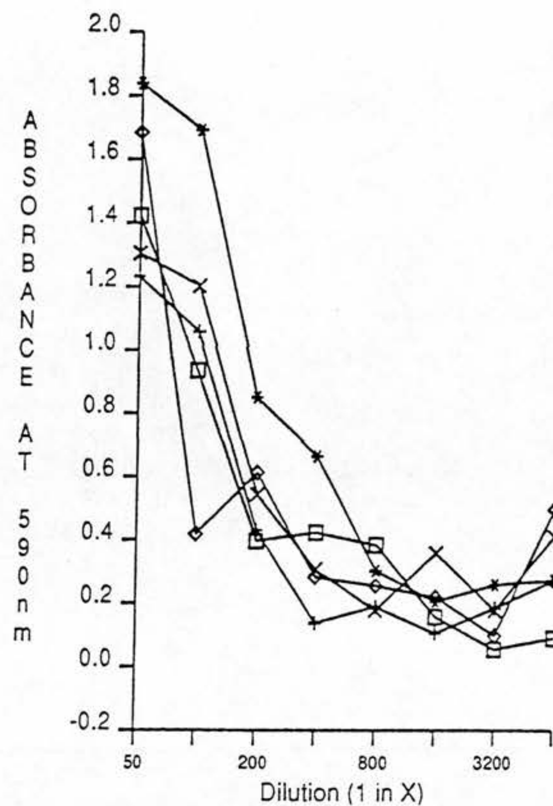
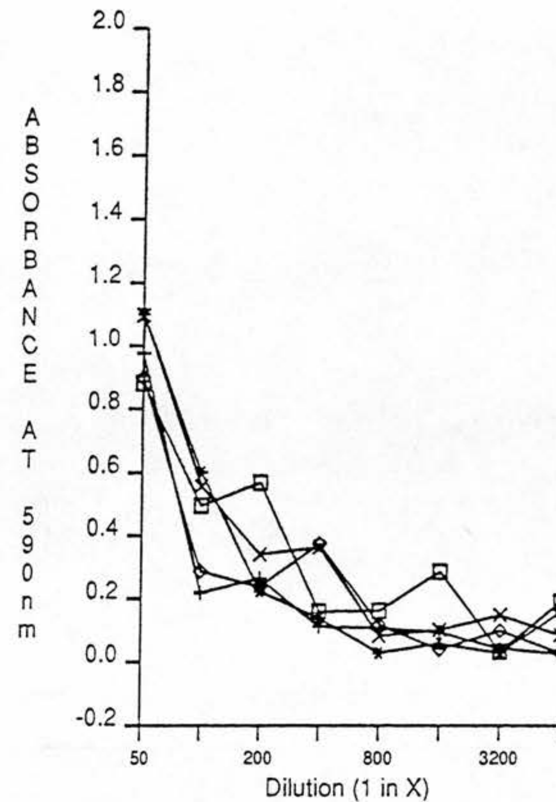
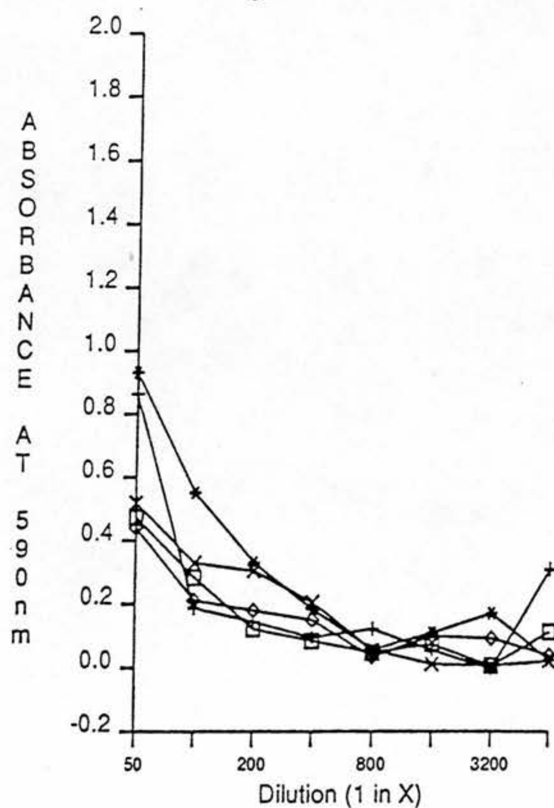


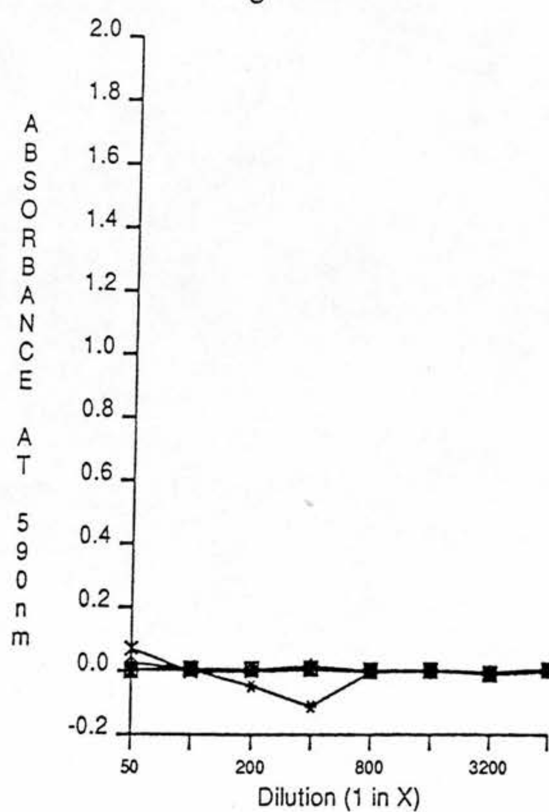
FIGURE 3:18. Binding activities of GL- serum against five antigens in ELISA before absorption and after four consecutive absorptions with heat-killed _S. typhimurium_ R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.











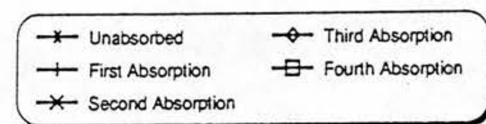
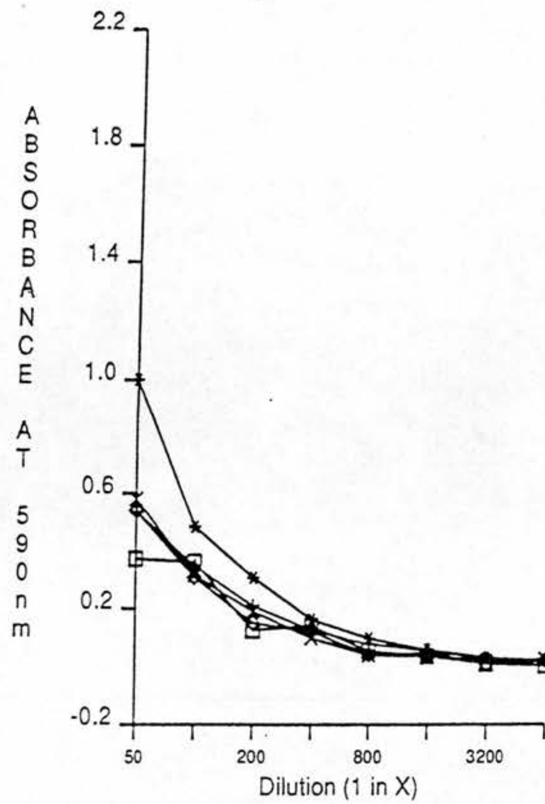
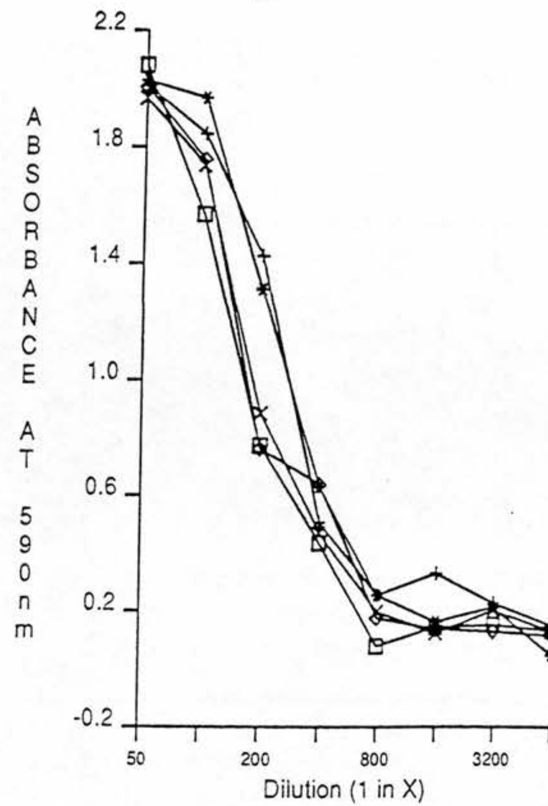
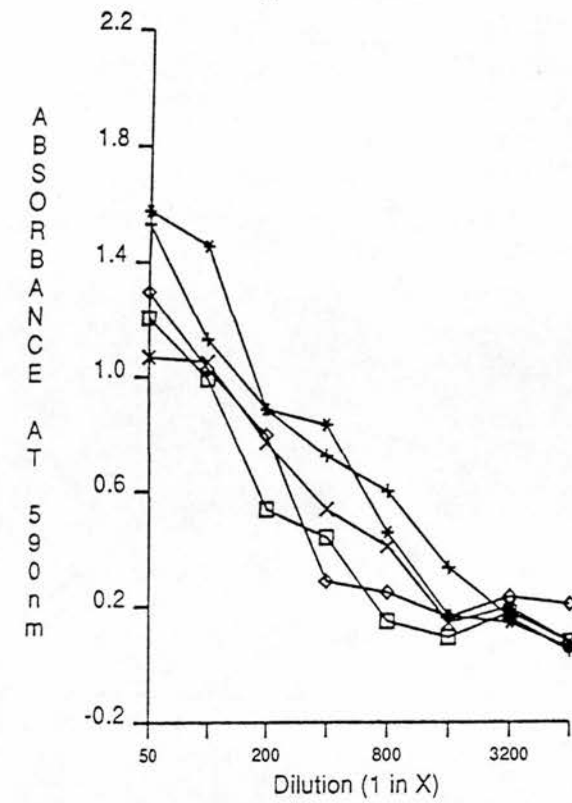
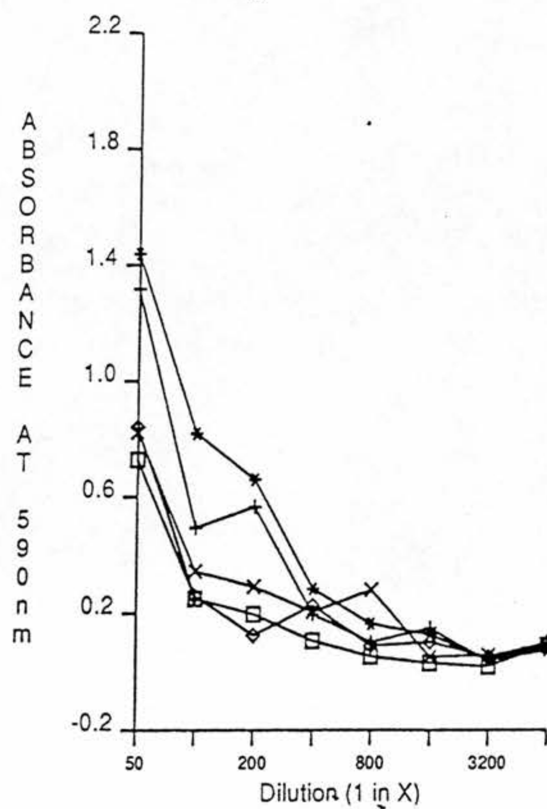


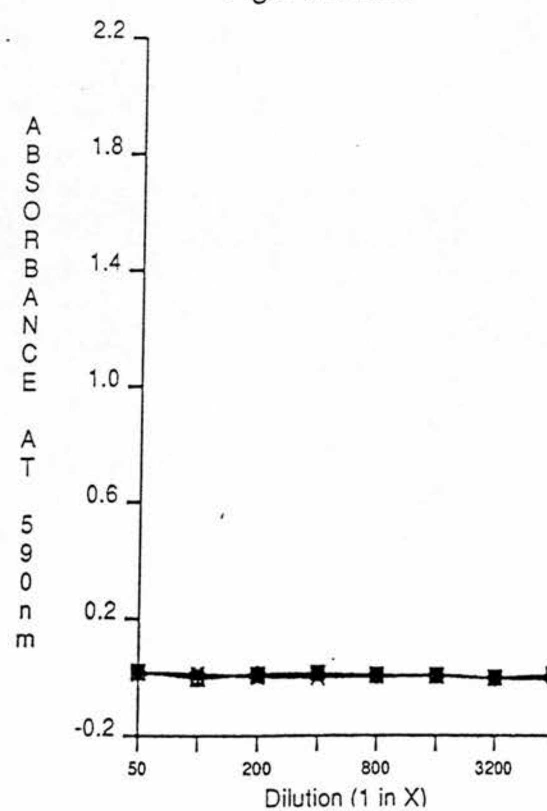
FIGURE 3:19. Binding activities of MED1 serum against five antigens in ELISA before absorption and after four consecutive absorptions with heat-killed *S. typhimurium* R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.











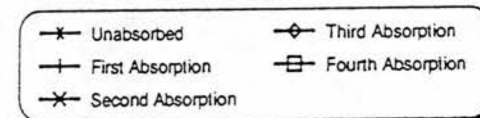


FIGURE 3:20. Binding activities of MED2 serum against five antigens in ELISA before absorption and after four consecutive absorptions with heat-killed S. typhimurium R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.

further reduction in IgG was obtained after the third absorption step had been performed.

b) Absorption of HNHS with viable bacterial cells.

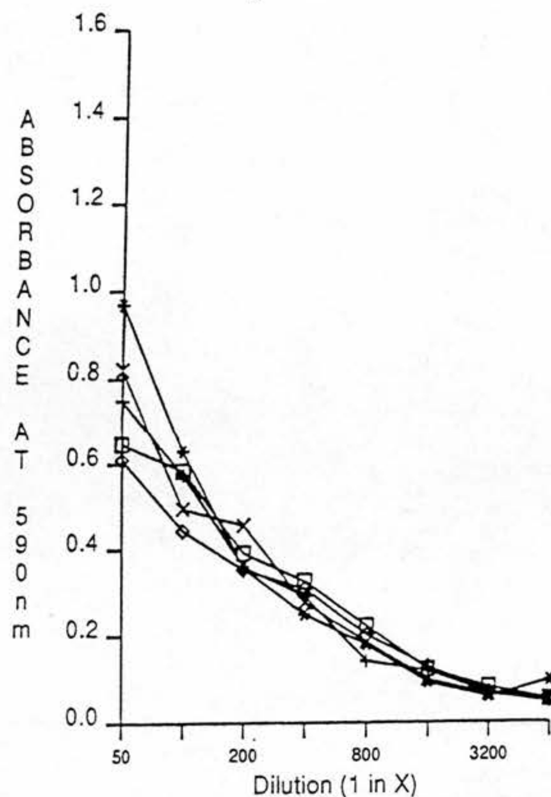
This produced the changes in IgG as shown in figures 3:21, 3:22, 3:23, and 3:24. Reactivity of heat-inactivated sera (before and after absorption) with ELISA antigens showed far greater fluctuation than fresh sera. Reduction in IgG levels at each step is noticeably less obvious than obtained above with the exception when purified LPS was used as antigen, although large fluctuations in IgG were noticeable.

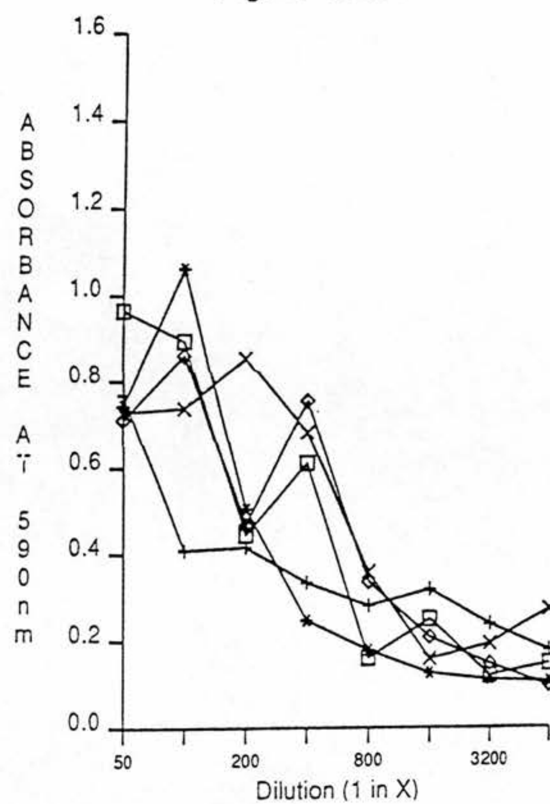
The ELISA strips containing only post-coated wells showed far higher absorbances at a serum dilution of 1:50 than obtained with fresh serum, but by a dilution of 1:100 this difference was removed and absorbances for HNHS were close to baseline.

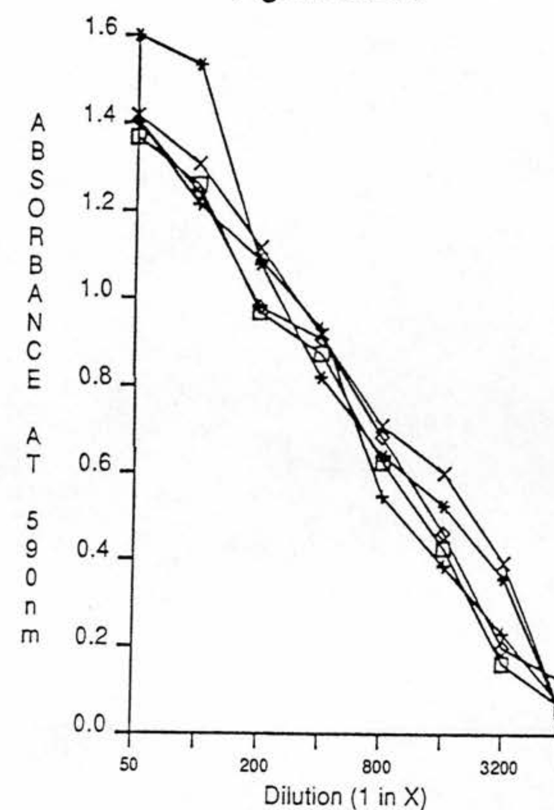
3:4:3. Inhibitory Activity of Soluble LPS-containing Antigens on the Binding of Anti-LPS IgG in ELISA.

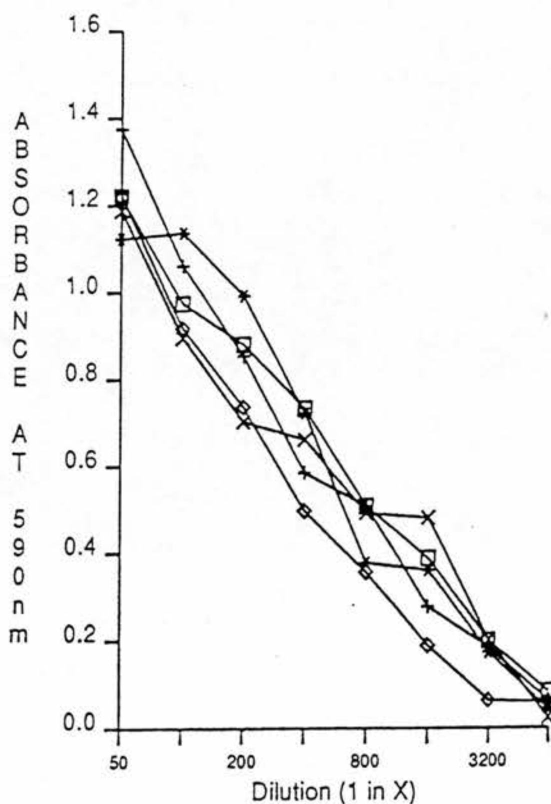
a) Effect of time of incubation of IgG and inhibitor.

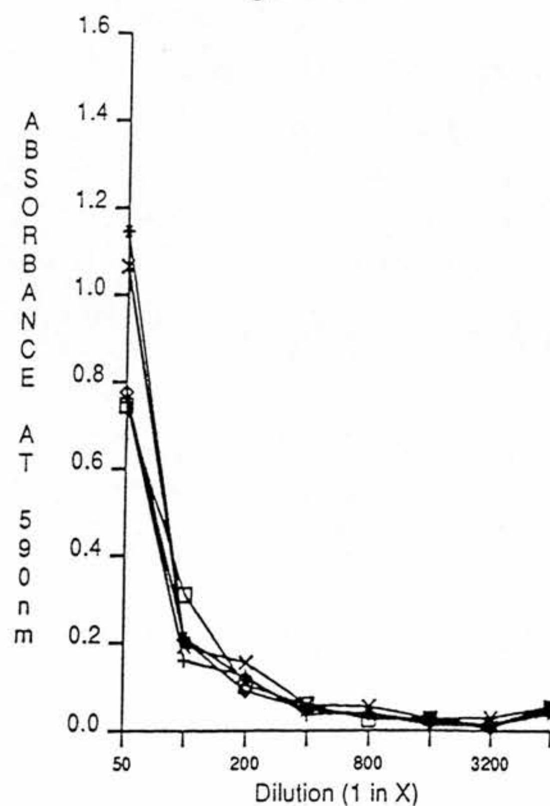
Inhibition of GL+ NHS was assayed as described in MATERIALS AND METHODS. GL+ at a final dilution of 1:100 was incubated at room temperature with doubling dilutions of inhibitor (S. typhimurium R878 LPS) from 8 times the concentration of carbohydrate initially loaded into ELISA strips for coating (i.e. doubling series of inhibitor from 1.6×10^{-2}mM carbohydrate per well). Samples of inhibitor-NHS mixture were removed at 10min intervals for assay in ELISA against purified LPS. Absorbances obtained were graphed in two ways: i) concentration of inhibitor versus absorption for each time point (figure 3:25a), and ii) time of co-incubation versus











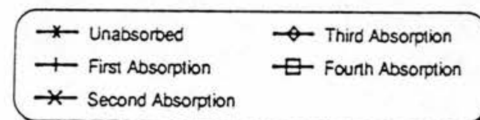


FIGURE 3:21. Binding activities of heat-inactivated GL+ serum against five antigens in ELISA before absorption and after four consecutive absorptions with viable S. typhimurium R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control contining no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.

Figure 3:22a

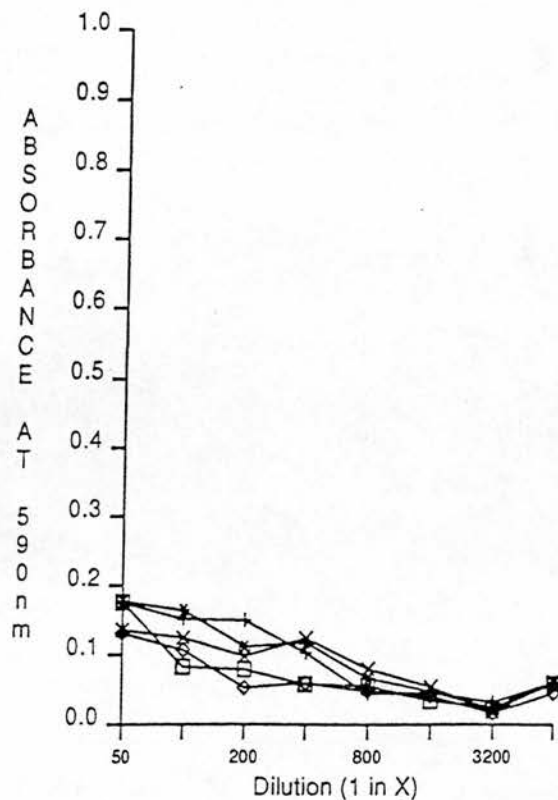


Figure 3:22b

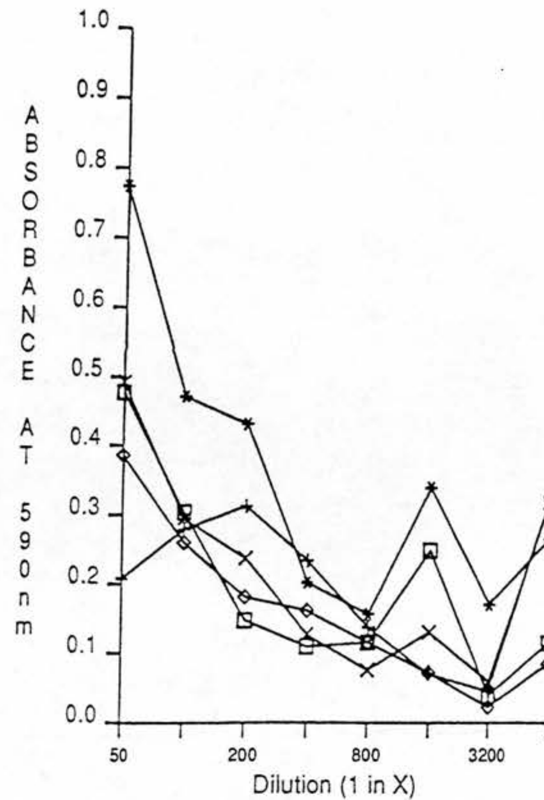


Figure 3:22c

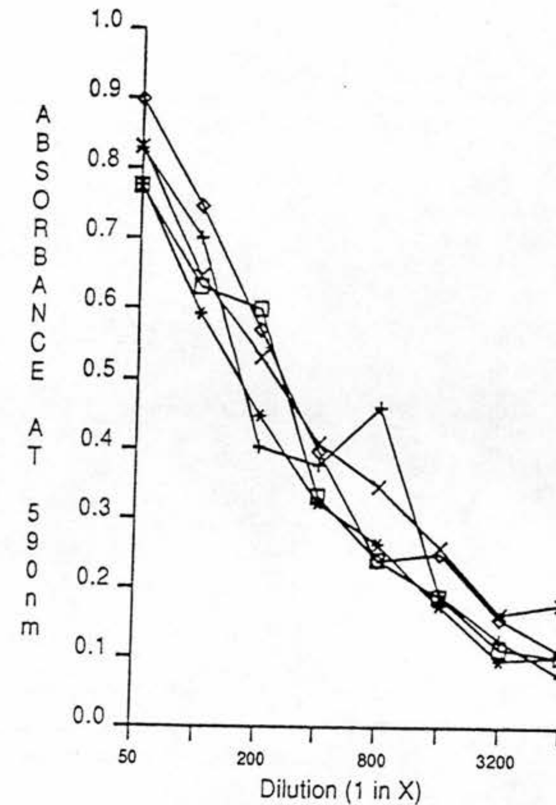
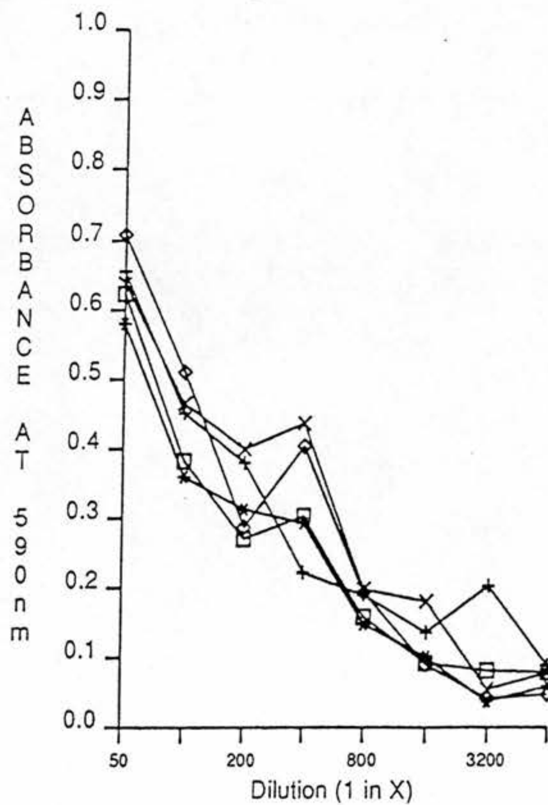


Figure 3:22d



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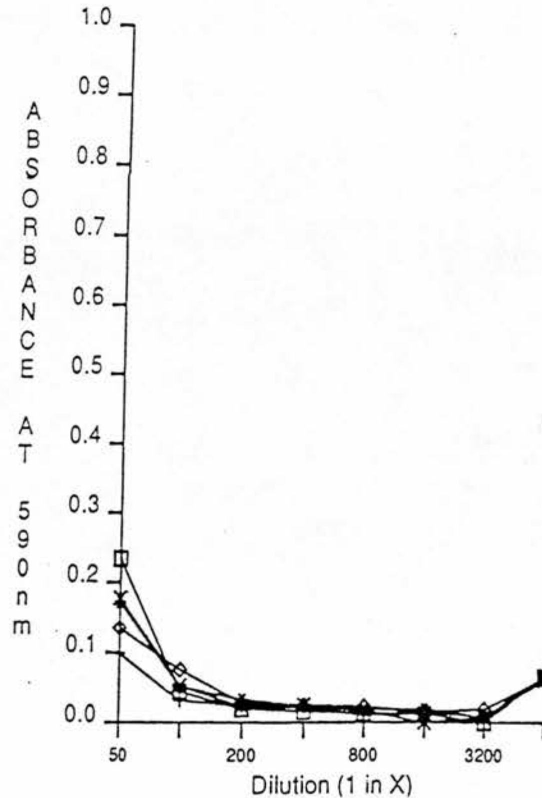


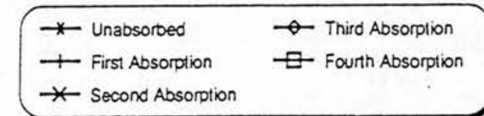


FIGURE 3:22. Binding activities of heat-inactivated GL- serum against five antigens in ELISA before absorption and after four consecutive absorptions with viable S. typhimurium R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.

Figure 3:23a

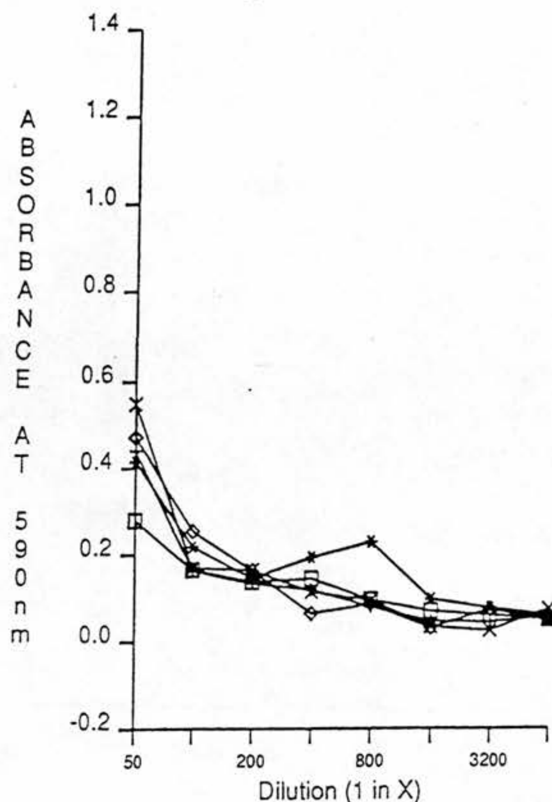


Figure 3:23b

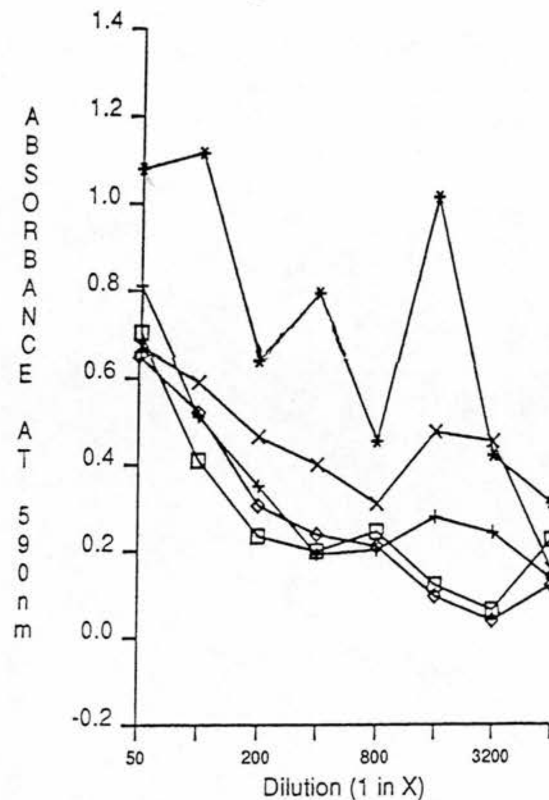


Figure 3:23c

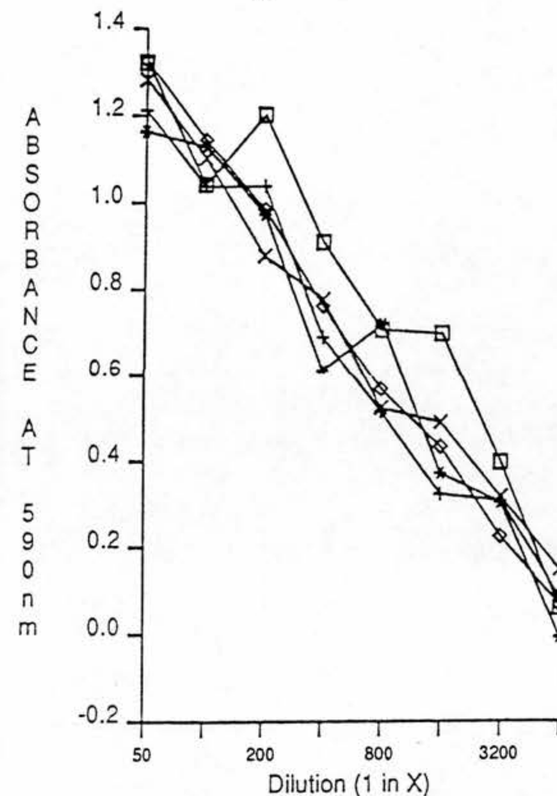


Figure 3:23d

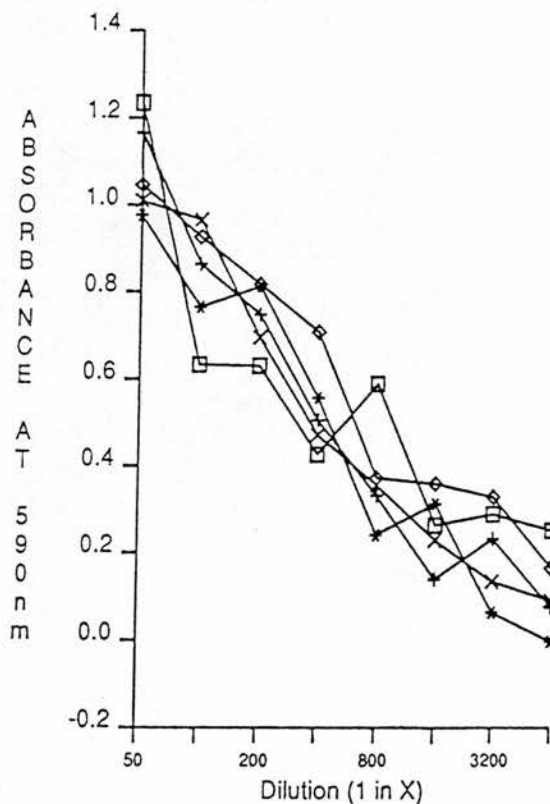
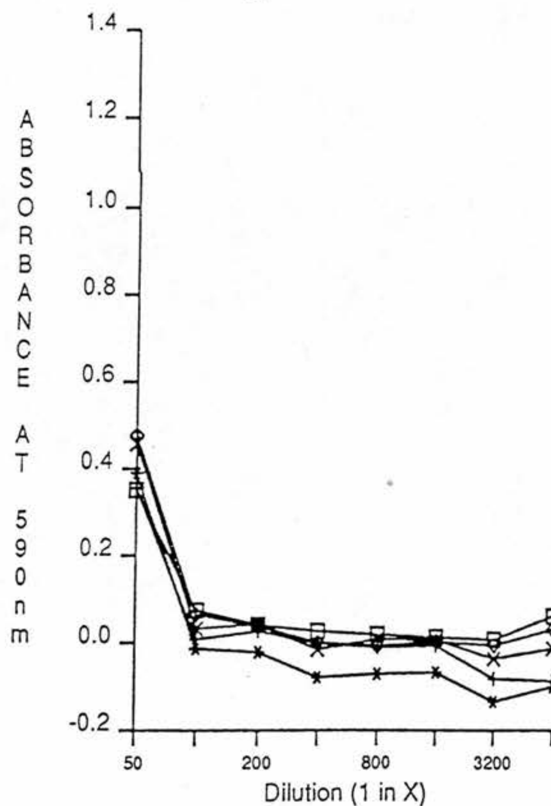


Figure 3:23e



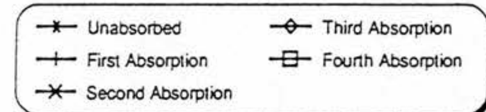
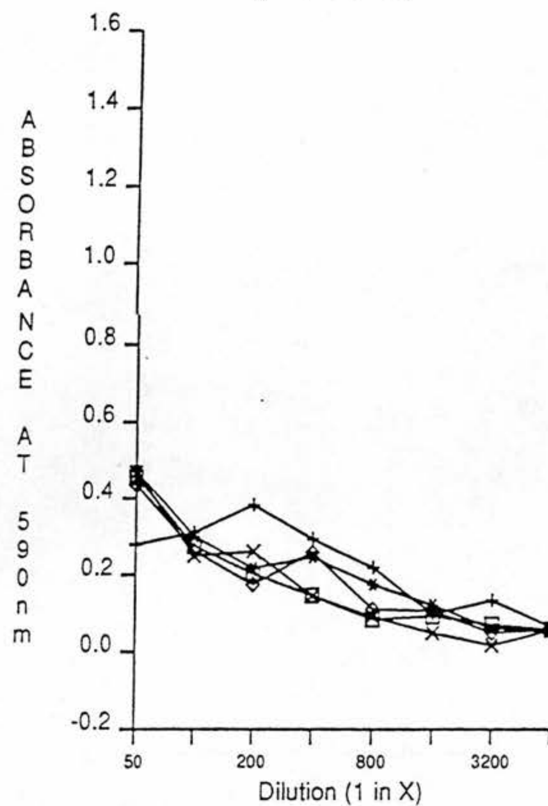
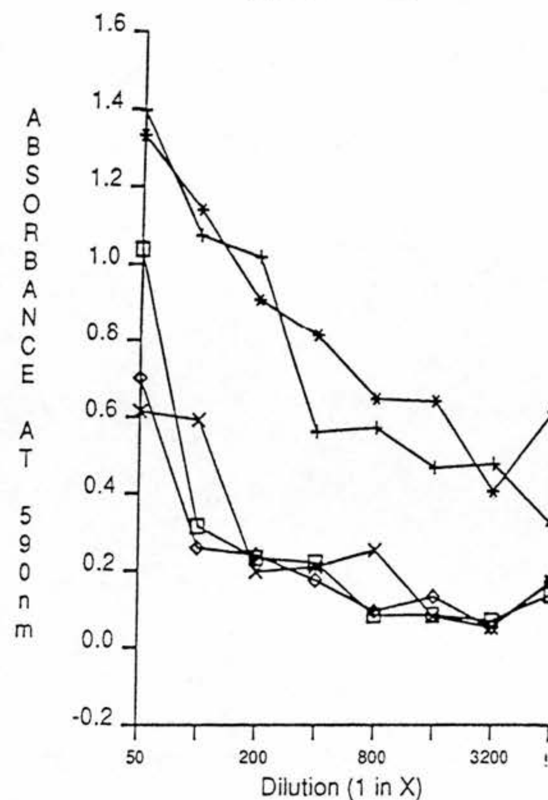
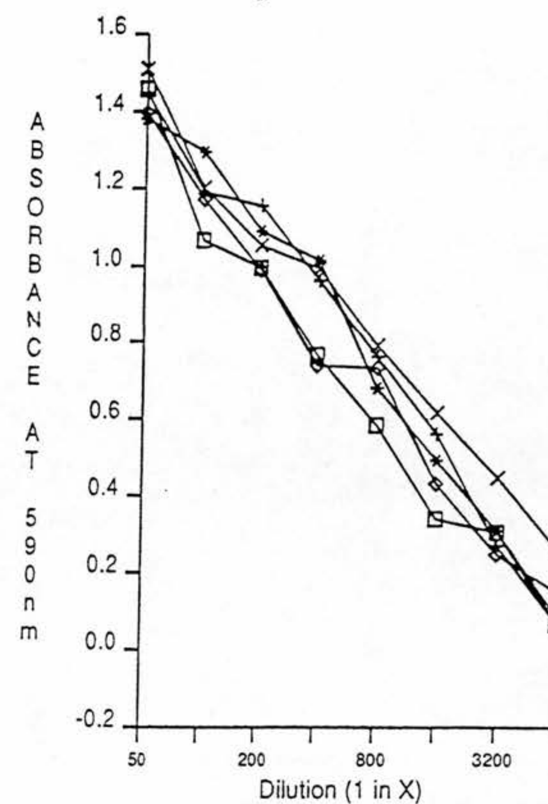
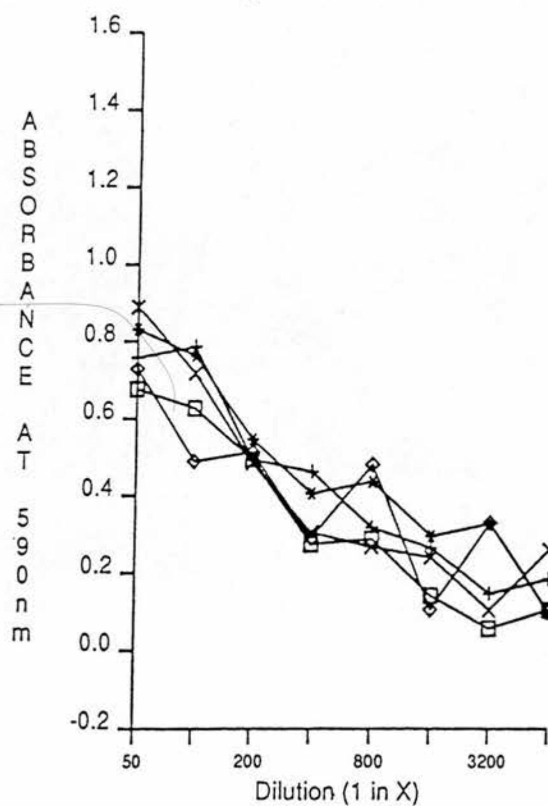


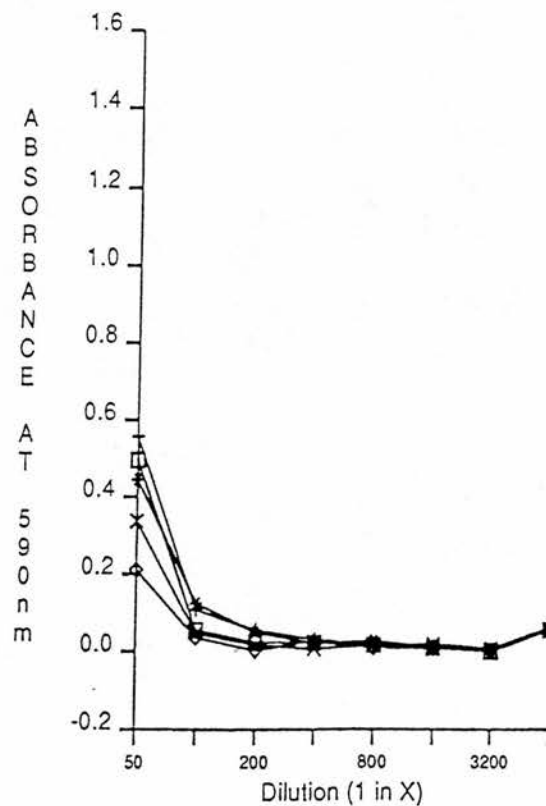
FIGURE 3:23. Binding activities of heat-inactivaated MED1 serum against five antigens in ELISA before absorption and after four consecutive absorptions with viable S. typhimurium R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.











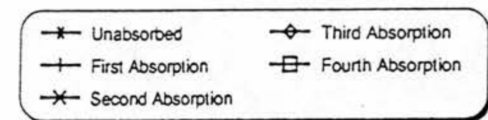


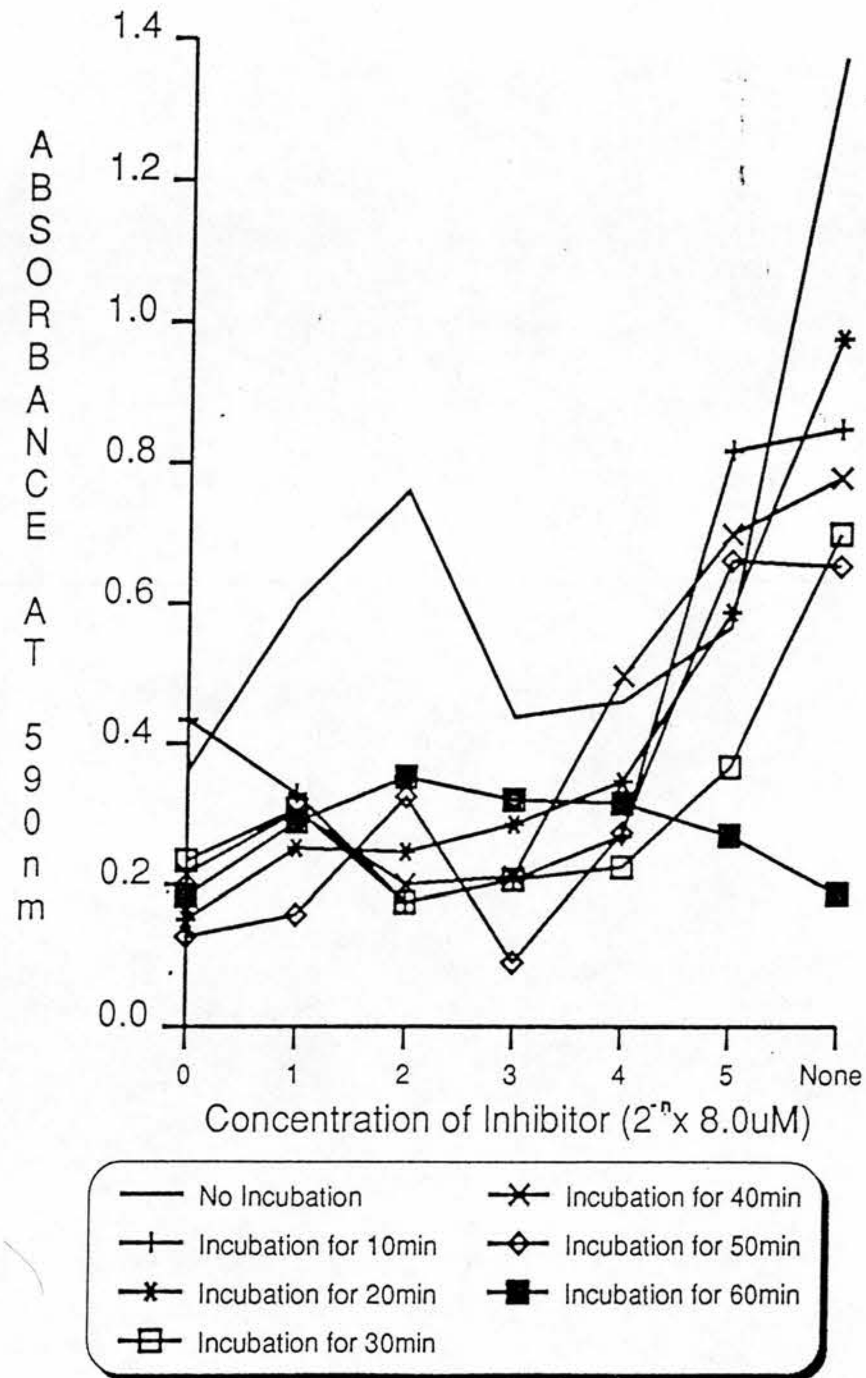
FIGURE 3:24. Binding activities of heat-inactivated MED2 serum against five antigens in ELISA before absorption and after four consecutive absorptions with viable S. typhimurium R878 bacteria. A doubling dilution series of serum from 1:50 to 1:3200 plus a control containing no serum was used. Figures (a), (b), (c), (d), and (e) represent results obtained with LPS-polymyxin complexes, uncomplexed LPS, outer membrane, heat-killed bacteria and blank solid phase antigens respectively.

absorbance for each concentration of inhibitor (figure 3:25b).
All time points produced greatest absorbance with no inhibitor present, with the exception of the sample removed after 60min co-incubation of inhibitor with GL+, which showed only very low levels of IgG. All other time points showed rapidly declining reactivities (i.e. increasing inhibition) with increasing concentration of inhibitor. At a concentration of 4.0×10^{-3}m$\underline{M}$ carbohydrate (2x concentration in well), however, a rise in absorbance was obtained for 0min pre-incubation, though at lower concentrations the absorbance again fell.

As inhibition could be observed in ELISA with no prior incubation of antibodies and inhibitor, a series of inhibitions was performed in this manner i.e. inhibitor and NHS were mixed *in situ* in ELISA microplates, then incubated for 60min to permit binding of antibodies to inhibitor and solid-phase antigen. The same serum (GL+) was used throughout because of its high reactivity in ELISA to all five antigenic preparations.

3:4:4. Activities of Three Inhibitors on Binding of IgG to ELISA Antigens.

Lipopolysaccharide and outer membrane (OM) were prepared in a 7 step doubling dilution curve from 8 times carbohydrate concentration in well (1.6×10^{-2}m$\underline{M}$ downwards) for use as inhibitors. Complexes of LPS with polymyxin were prepared in a doubling dilution series from twice the concentration in the well (8.0×10^{-3}m$\underline{M}$ carbohydrate). The appropriate concentration of inhibitor (50ul) and GL+ at a dilution of 1:50 (50ul) were placed in microplate wells containing each of



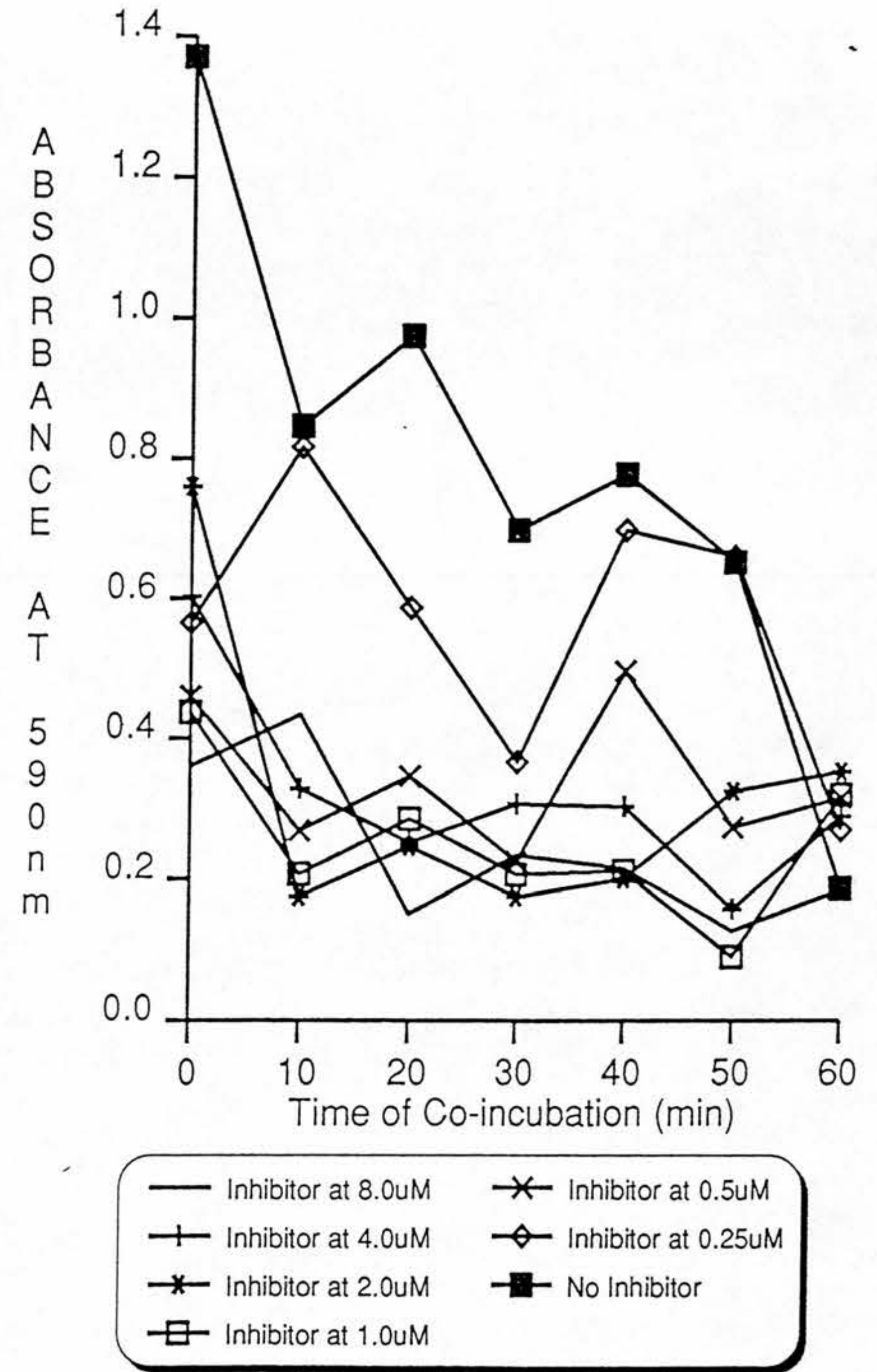


FIGURE 3:25. Inhibition of ELISA reactivity of GL+ serum against <u>S. typhimurium</u> R878 LPS with purified LPS from the same organism. Figure (a) represents the activities obtained with a range of inhibitor concentrations for each period of co-incubation (as described in the key), and figure (b) represents the activities obtained with each concentration of inhibitor (as described in the key) after each period of co-incubation.

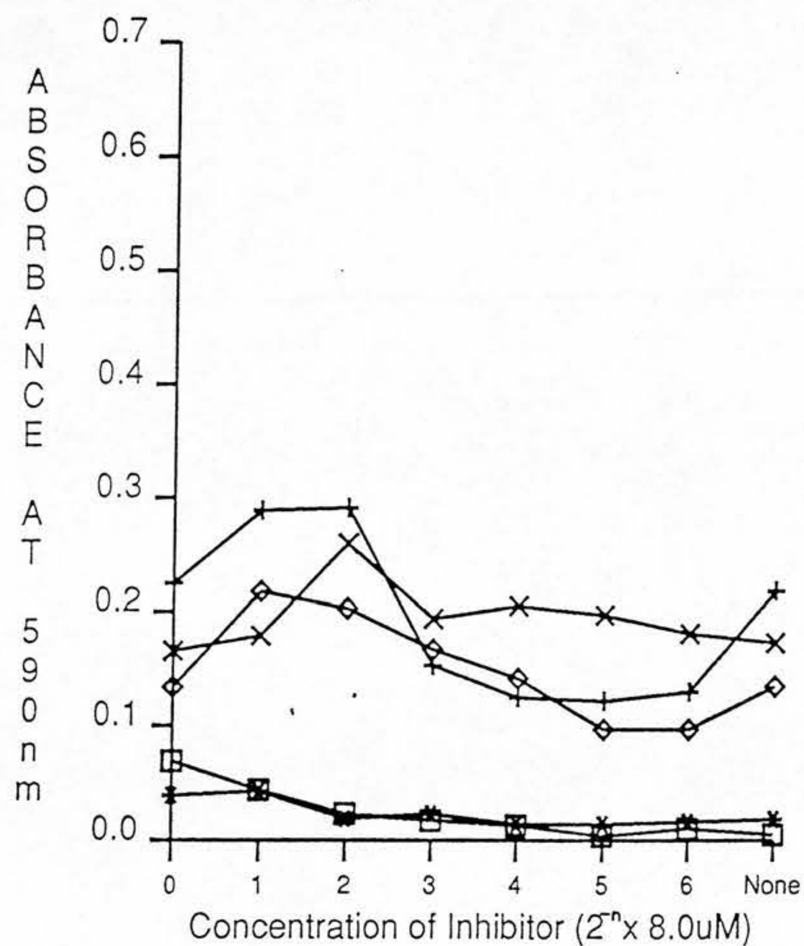
the antigenic preparations. This gave a final serum dilution of 1:100, and a dilution series of inhibitor from 8.0×10^{-3}mM carbohydrate for LPS and OM, and from 4.0×10^{-3}mM for LPS-polymyxin. After 60 min incubation, strips were washed and incubated for 60 min with 100ul of a 1:500 dilution of anti-human IgG-urease conjugate. Further washing was carried out and substrate was added to wells (100ul). Absorbances were read at 405nm.

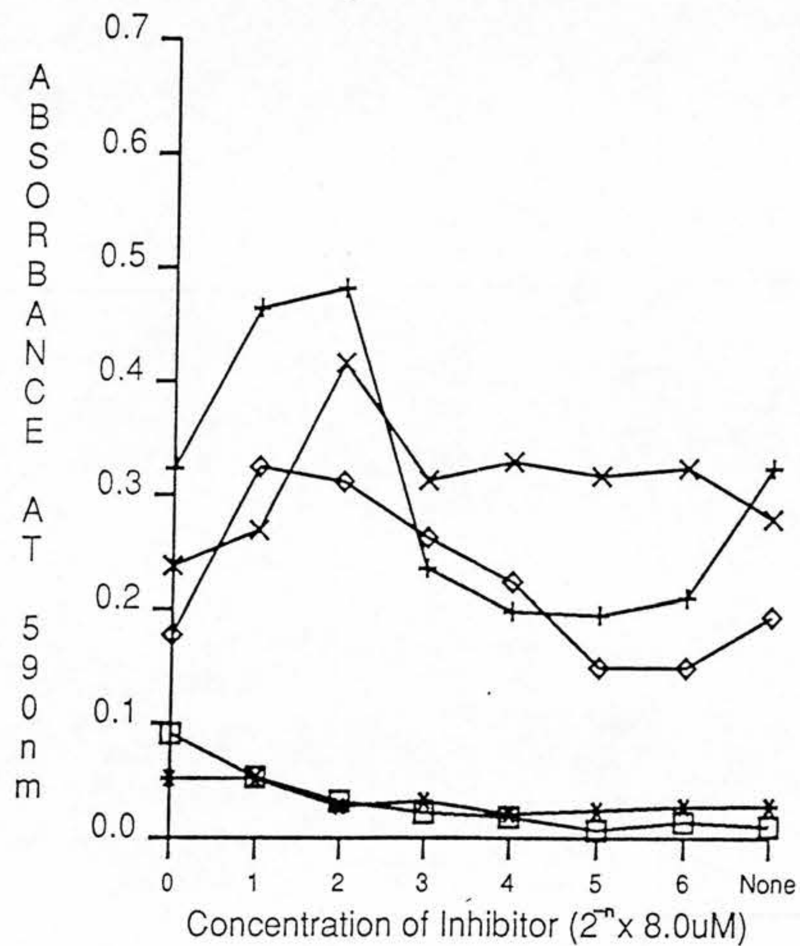
a) Inhibition with uncomplexed LPS:

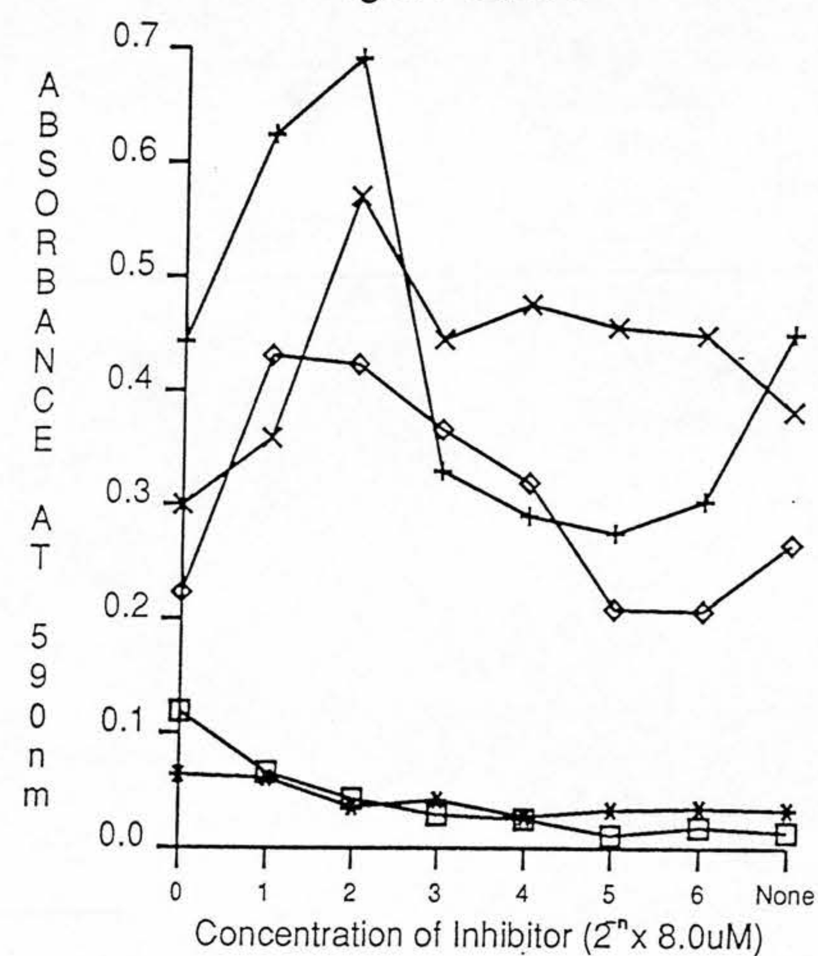
Results obtained after 30, 45, and 60 min colour development (figures 3:26a-c) indicate that results were similar at all times and that addition of purified LPS to serum inhibited the binding activity of antibodies to lipopolysaccharide itself and to heat-killed organisms, but not to outer membrane. The blank control strips showed only neligible binding by NHS. Very low levels of binding were seen to LPS-polymyxin complex even in absence of inhibitor, thus ELISA results for this antigen must be disregarded. Inhibition was obtained only between 1.0×10^{-3} and 0.125×10^{-3} mM inhibitor against LPS, and between 0.5×10^{-3} and 0.125×10^{-3}mM inhibitor against bacteria. Above these concentrations binding to LPS and bacteria was greater than when no inhibitor was present. This reflects the previous inhibition results (figure 3:25). At concentrations of greater than 2.0×10^{-3}mM and 4.0×10^{-3}mM against LPS and bacteria respectively, results show a drop towards inhibition once again.

b) Inhibition with outer membrane (OM).

The greatest reduction in absorbance at all time points was obtained versus LPS and OM at all concentrations of inhibitor (figure 3:27a-c). The results for blank strips remains on the axis







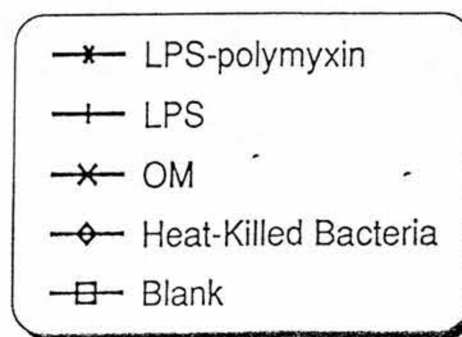
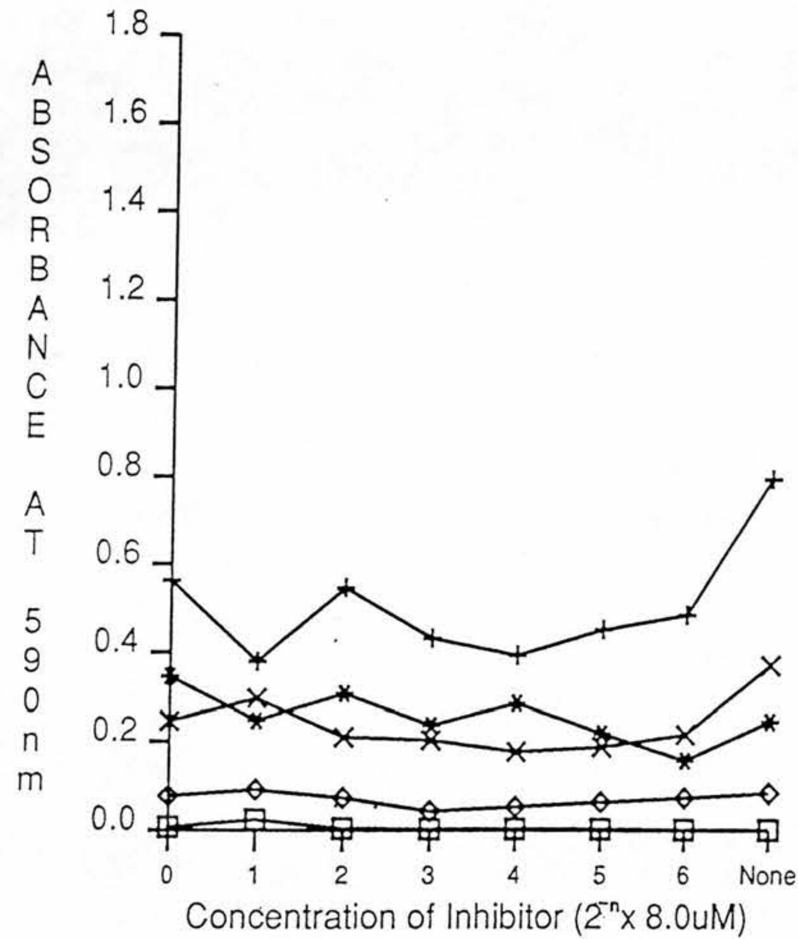
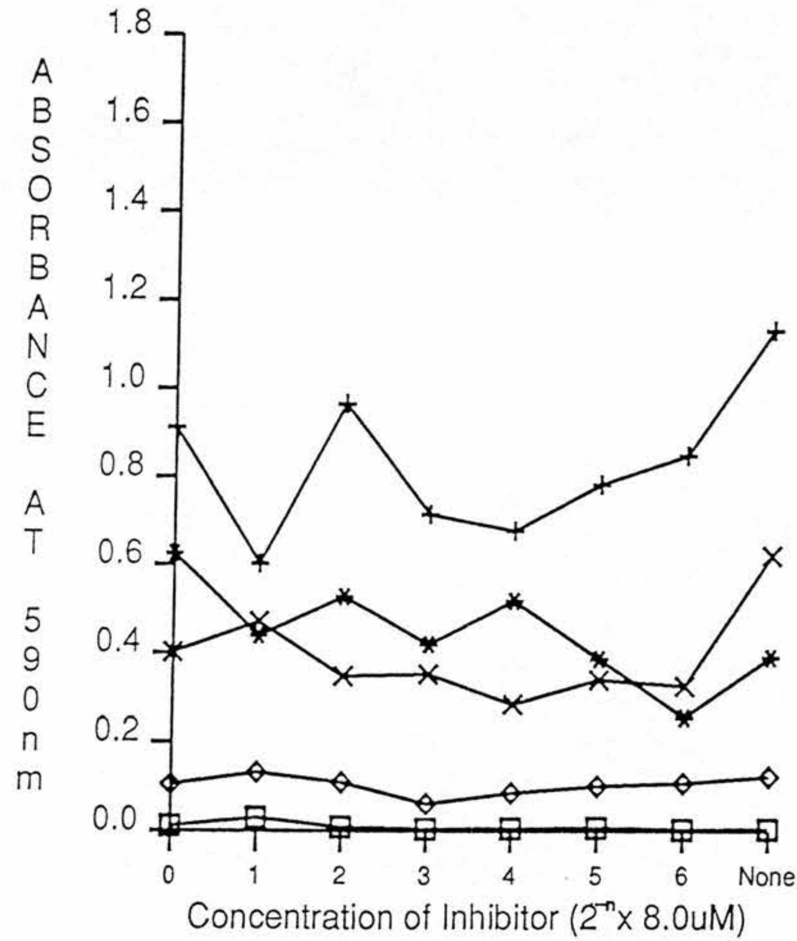
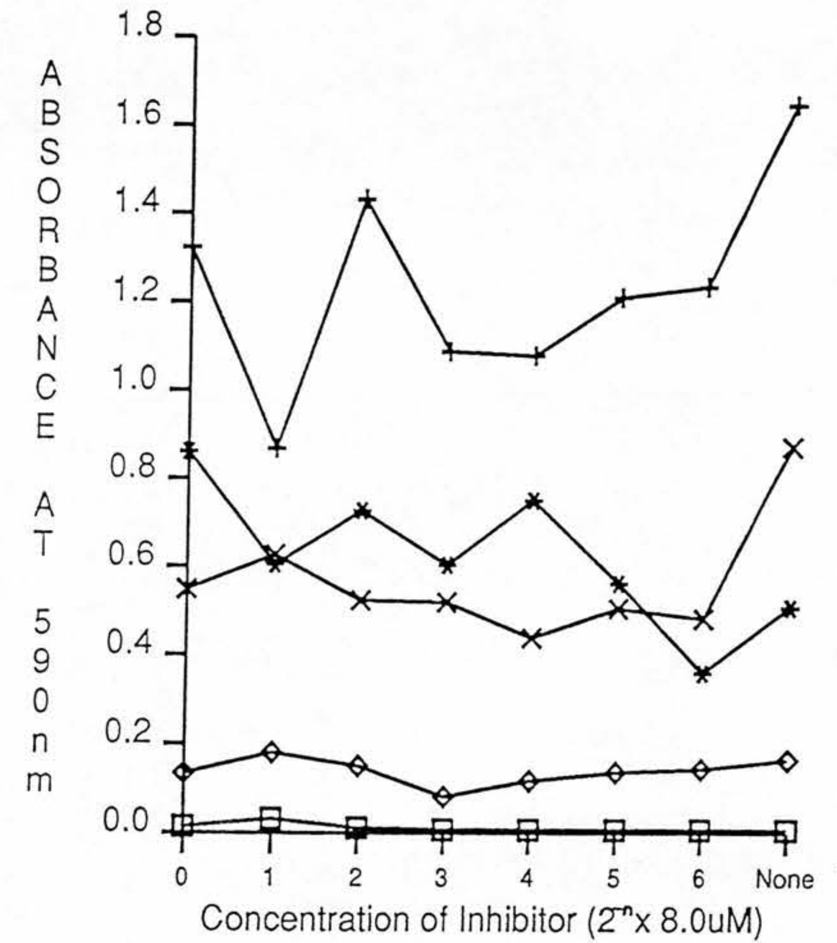


FIGURE 3:26. Inhibition of ELISA reactivity of GL+ serum against four *S. typhimurium* R878 LPS-containing antigens and a blank (as described in the key) with a doubling dilution series of purified LPS from this organism and a control containing no inhibitor. Colour development was carried out for 30min (figure a), 45min (figure b) and 60min (figure c).







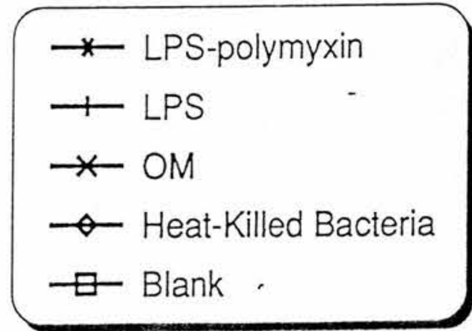
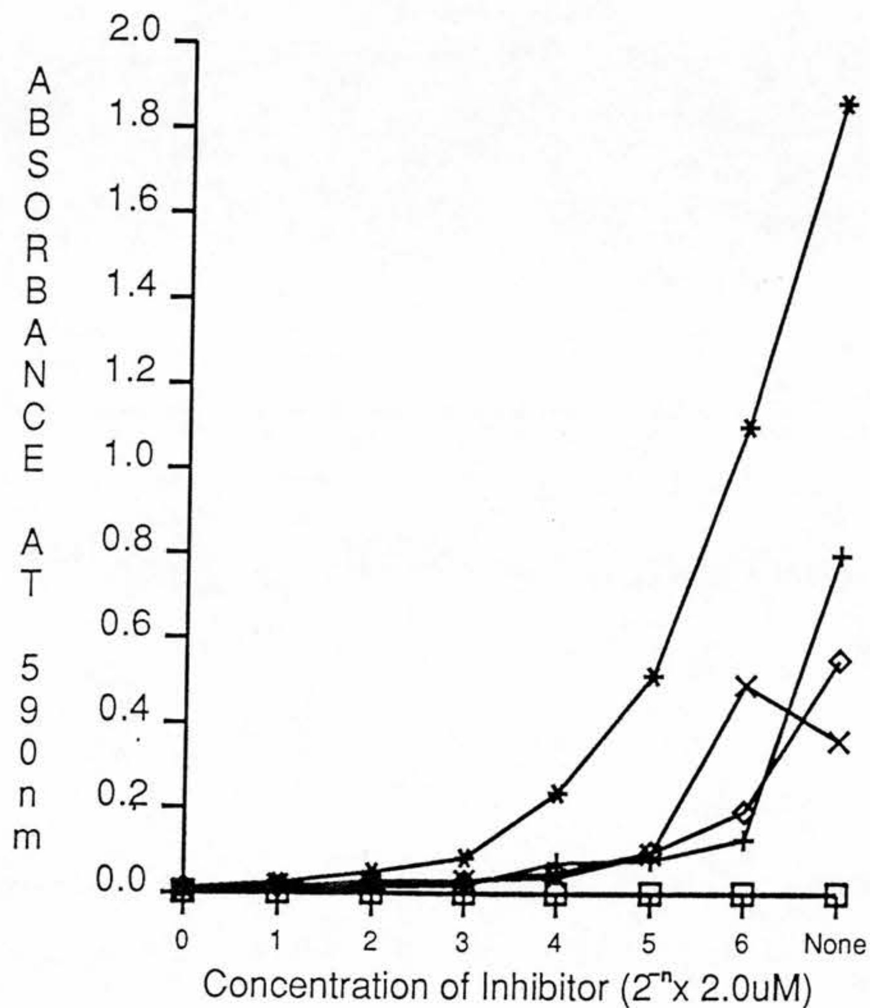
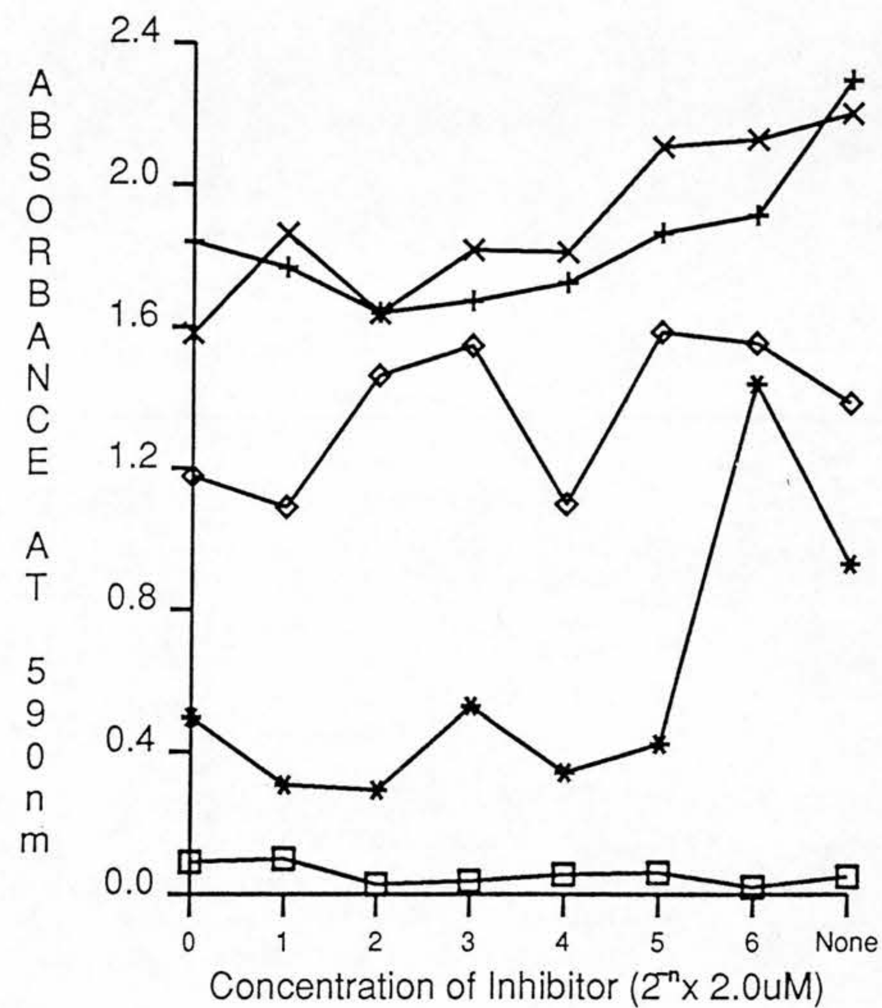


FIGURE 3:27. Inhibition of ELISA reactivity of GL+ serum against four S. typhimurium R878 LPS-containing antigens and a blank (as described in the key) with a doubling dilution series of outer membrane from this organism and a control containing no inhibitor. Colour development was carried out for 30min (figure a), 45min (figure b) and 60min (figure c).





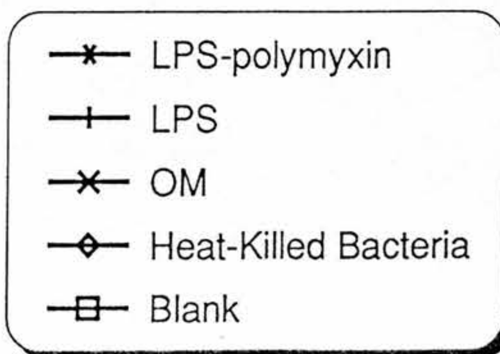


FIGURE 3:28. Inhibition of ELISA reactivity of GL+ serum against four S. typhimurium R878 LPS-containing antigens and a blank (as described in the key) with a doubling dilution series of LPS-polymyxin complexes from this organism and a control containing no inhibitor. Figures (a) and (b) represent repeat assays carried out under indentical conditions.

of the graph. Absorbances versus bacteria varies little over the range of concentrations of inhibitor. With LPS-polymyxin only one concentration (0.125×10^{-3}m$\underline{M}$) produced an absorbance lower than in absence of inhibitor. Other points against LPS-polymyxin show large deviations.

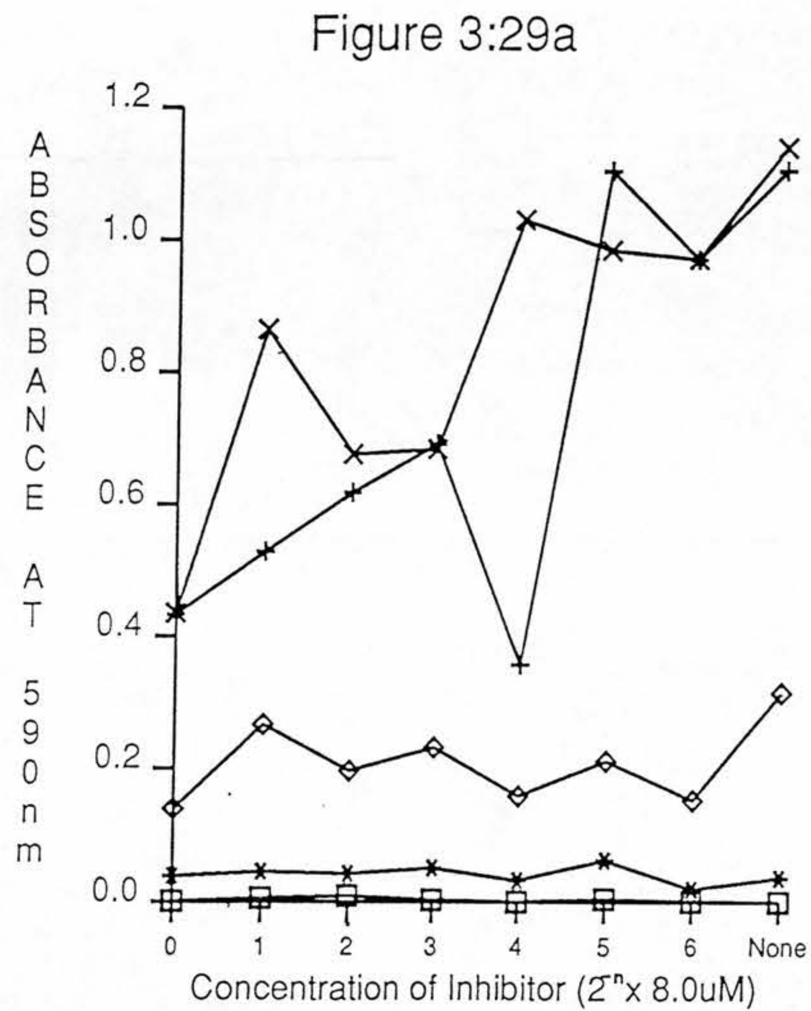
c) Inhibition with LPS-polymyxin complexes.

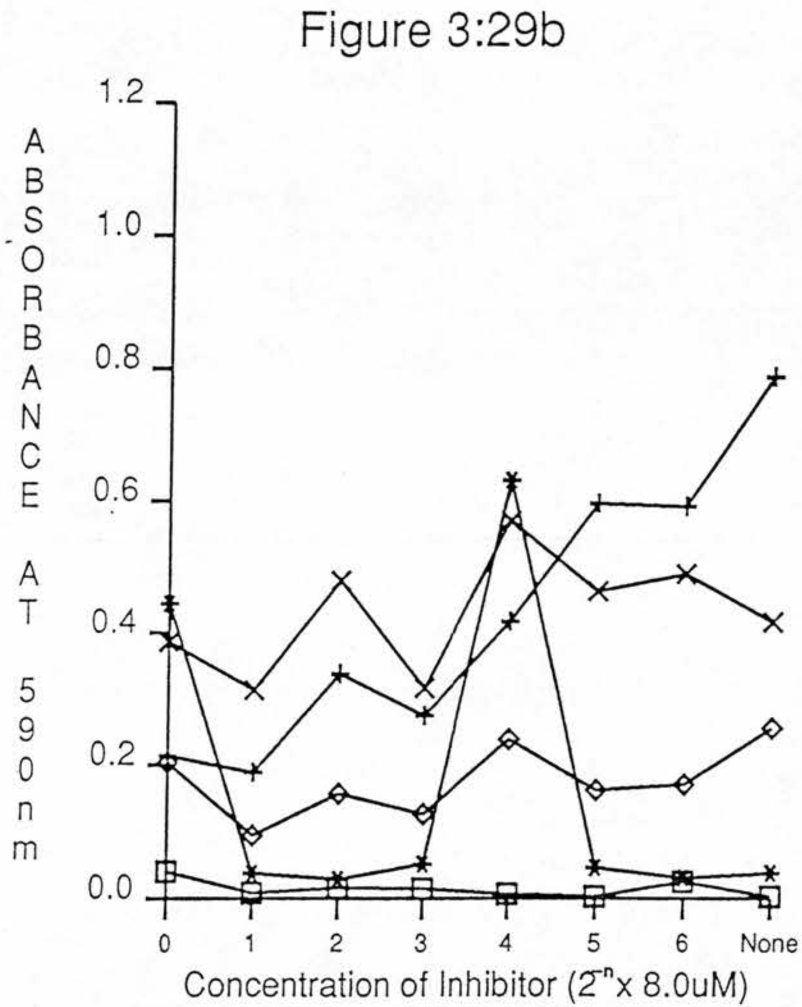
1. New strips containing LPS-polymyxin were prepared for this in view of the low absorbances obtained above. For this, LPS-polymyxin complex at a final concentration of 4.0×10^{-3}m$\underline{M}$ was used for coating strips. Inhibitor was therefore produced at a final concentration in wells from 4.0×10^{-3}m$\underline{M}$ in a doubling dilution series.

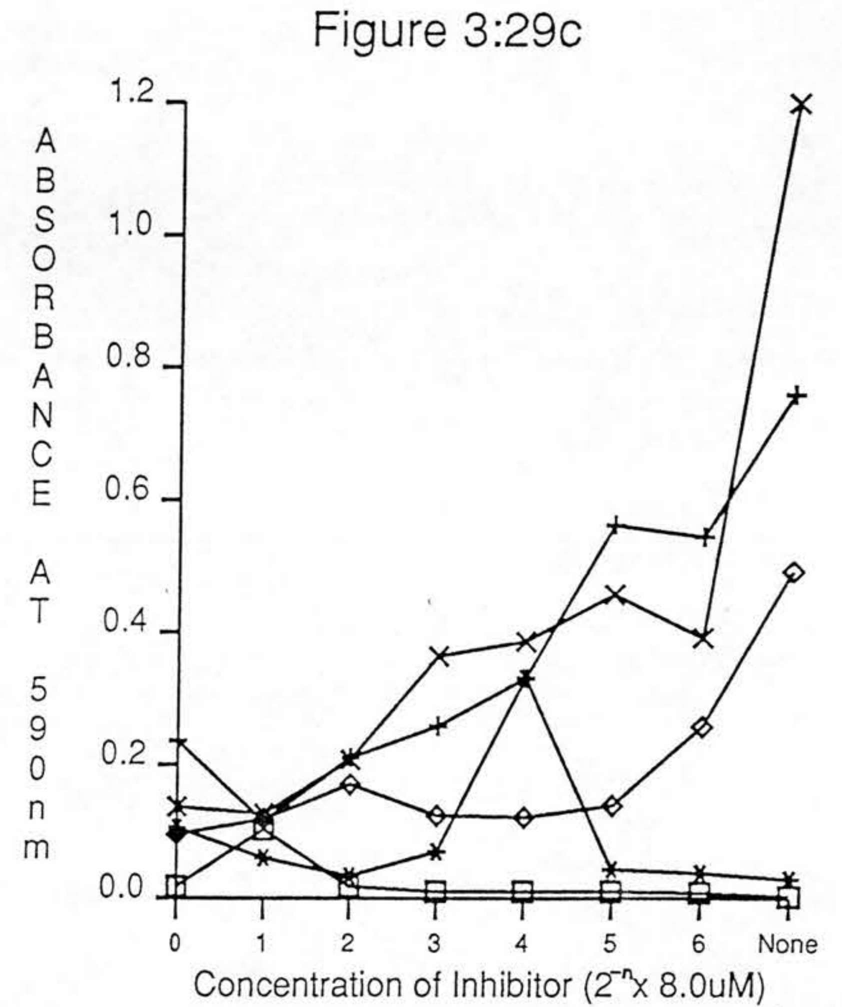
Inhibition was clearly demonstrable versus all four antigens (figure 3:28a). The greatest inhibition occurred against LPS-polymyxin, and significant inhibition was also observed versus LPS, bacteria, and OM for all concentrations of inhibitor.

2. Repetition of this inhibition in an identical manner produced the results indicated in figure 3:28b. Although the results show inhibition of binding of IgG to all antigens, the absorbance values and position if the curves is at variance with those in figure 3:28a. Once again inhibition was most noticeable with LPS-polymyxin coated wells, although with inhibitor at a concentration of 3.125×10^{-5}m$\underline{M}$ absorbance was higher than with no inhibitor present. Inhibition versus LPS and OM also occurred, again at significant levels. With heat-killed bacteria as the solid-phase antigen, much variation was obtained between consecutive points on the graph, with some lying above and some lying below the absorbance where no inhibitor is present.

d) Inhibition was repeated once more with each of the three







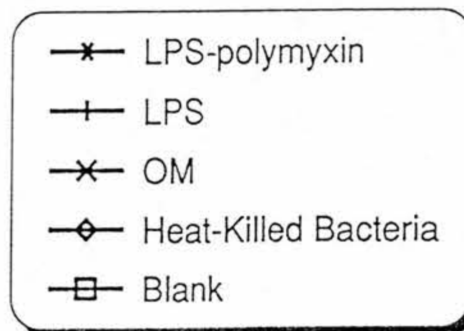


FIGURE 3:29. Inhibition of ELISA reactivity of GL+ serum against four *S. typhimurium* R878 LPS-containing antigens and a blank (as described in the key) with doubling dilution series of three inhibitors. Figures (a), (b) and (c) represent the results obtained by inhibition with LPS-polymyxin complexes, uncomplexed LPS and outer membrane respectively.

inhibitors against the five solid-phase antigens (LPS, LPS-polymyxin, OM, bacteria, and blank). Inhibitors were prepared from 8.0×10^{-3}m$\underline{M}$ in a doubling dilution series. Results are presented in figures 3:29a-c as absorbance versus dilution of inhibitor. These indicate variance from results obtained previously. With each inhibitor, LPS-polymyxin coated strips showed absorbances approaching baseline values with the exception of three points (at 0.5×10^{-3} and 8.0×10^{-3}m$\underline{M}$ for LPS and at 0.5×10^{-3}m$\underline{M}$ versus OM). Reactivity of NHS with bacteria showed much variability in absence of inhibitor. The results obtained for the final series of inhibitions can be summarised as follows:

i) LPS-polymyxin complexes, with both LPS and OM, showed decreases in antibody binding to LPS and OM with increasing concentration of inhibitor. All dilutions of LPS-polymyxin produced small decreases in absorbance versus bacteria. Binding of antibodies to LPS in absence of inhibitor produced a far higher absorbance (1.10) than against LPS in the other two assays (0.78 and 0.76).

ii) Uncomplexed LPS showed inhibition of antibody reactivity with LPS, OM and bacteria. Marked inhibition occurred to LPS. Lower concentrations of inhibitor showed some inhibitory activity versus OM, but large fluctuations were obtained in absorbance between consecutive points. Similarly, inhibition of binding of antibodies to bacteria occurred, but only at a very low level. The increase in binding to solid phase antigen previously obtained with high LPS inhibitor concentrations (figures 3:25 and 3:26) was less noticeable in this experiment. Reactivity of serum in absence of inhibitor was low versus OM (0.416) in comparison with the other two inhibitions (with values of 1.138 and 1.231).

iii) OM produced a similar reduction in binding activity to LPS with increasing concentration. Very high binding of GL+ to OM in absence of inhibitor was obtained (1.23 compared with 0.42 and 0.46 for LPS and LPS-polymyxin respectively), thus this may be due to the presence of a contaminant in this well. Strong inhibitory activity of OM was clearly demonstrated versus OM, LPS and bacteria.

3:5. Alteration of Expression of Lipopolysaccharide Epitopes under Different Nutrient Conditions.

3:5:1. Effect of Nutrient Conditions upon LPS Expression.

a) *E. coli* O18:K1 was inoculated into 10ml nutrient broth and incubated at 37°C for 4h after which time 0.5ml was removed for inoculation into 10ml of the media detailed in table 3:2. Bacteria were cultured for 18h as described in MATERIALS AND METHODS, harvested by centrifugation (4000*g*, 10min, Heraeus Christ bactifuge) and washed twice in sterile PBS, then finally resuspended in 10ml of PBS. Absorbances of suspensions were measured in a spectrophotometer at 525nm (A_{525})and were tabulated (table 3:2). Concentrations of bacteria in several of the suspensions were adjusted to give an A_{525} of 0.5 - 0.6. Proteinase K digestions were performed on these, and lipopolysaccharides were visualised by PAGE (14% acrylamide separating gel) followed by silver staining. Results of silver staining are presented in figure 3:30.

The results in table 3:2 indicate that strong growth was observed in media numbers 1, 2, 3, 4, 6, 8 and 9. Less, though significant, growth occurred in all other media except number 12 (PBS).

Silver staining of PAG (figure 3:30) revealed little difference between high molecular weight bands of O-antigen. Heavier staining of the first major band of core, running at the front, was obtained with bacteria grown in the presence of serum at a concentration of 20% or greater in NB. Growth in NB and in NB-serum mixtures resulted in production of a minor band running in front of the first major core band. Expression of this component appeared to increase with increasing concentration of NB.

TABLE 3:2. Absorbance at 525nm after 18h of E. coli O18:K1 Grown in Different Nutrient Conditions.

No.	CONCENTRATION IN MEDIUM			A_{525}
	serum*	broth**	PBS	
1.	100%	-	-	1.37
2.	50%	50%	-	1.70
3.	50%	-	50%	1.10
4.	20%	80%	-	1.55
5.	20%	-	80%	0.75
6.	5%	95%	-	1.38
7.	5%	-	95%	0.35
8.	-	100%	-	1.48
9.	-	50%	50%	0.99
10.	-	20%	80%	0.447
11.	-	5%	95%	0.115
12.	-	-	100%	0.071

TABLE 3:3. Absorbance at 525nm after 18h of E. coli O18:K^- Grown in Different Nutrient Conditions.

No.	CONCENTRATION IN MEDIUM			A_{525}
	serum*	broth**	PBS	
1.	100%	-	-	1.06
2.	50%	50%	-	1.90
3.	50%	-	50%	0.92
4.	20%	80%	-	1.55
5.	20%	-	80%	0.67
6.	5%	95%	-	1.75
7.	5%	-	95%	0.45
8.	-	100%	-	1.75
9.	-	50%	50%	1.10
10.	-	20%	80%	1.02
11.	-	5%	95%	0.51
12.	-	-	100%	0.238

* Sheep serum inactivated by heating at 56-60°C for 45min, then stored at -20°C until required.

** Gibco nutrient broth

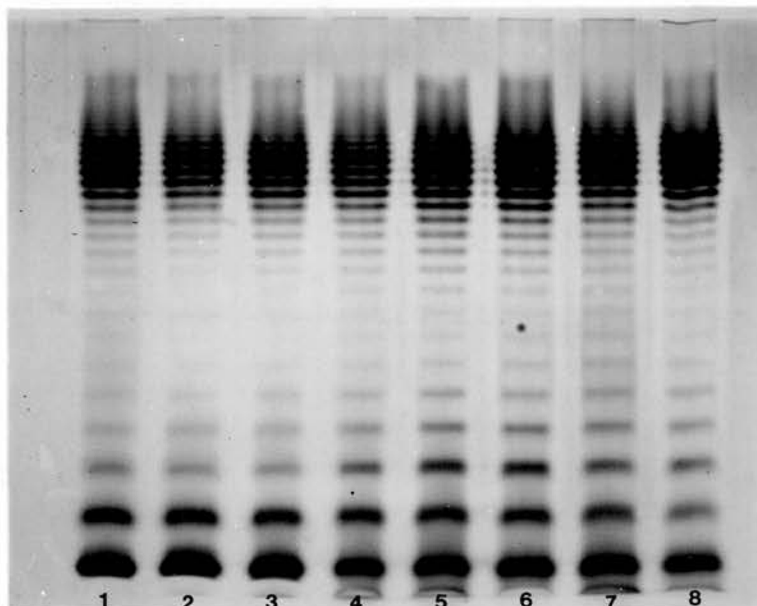


FIGURE 3:30. Silver stain of proteinase K digests from overnight cultures of E. coli O18:K1 grown in media as described in Table 3:2. Growth media were: track 1 - medium 1; track 2 - medium 2; track 3 - medium 4; track 4 - medium 6; track 5 - medium 8; track 6 - medium 9; track 7 medium 10; and track 8 - medium 11.

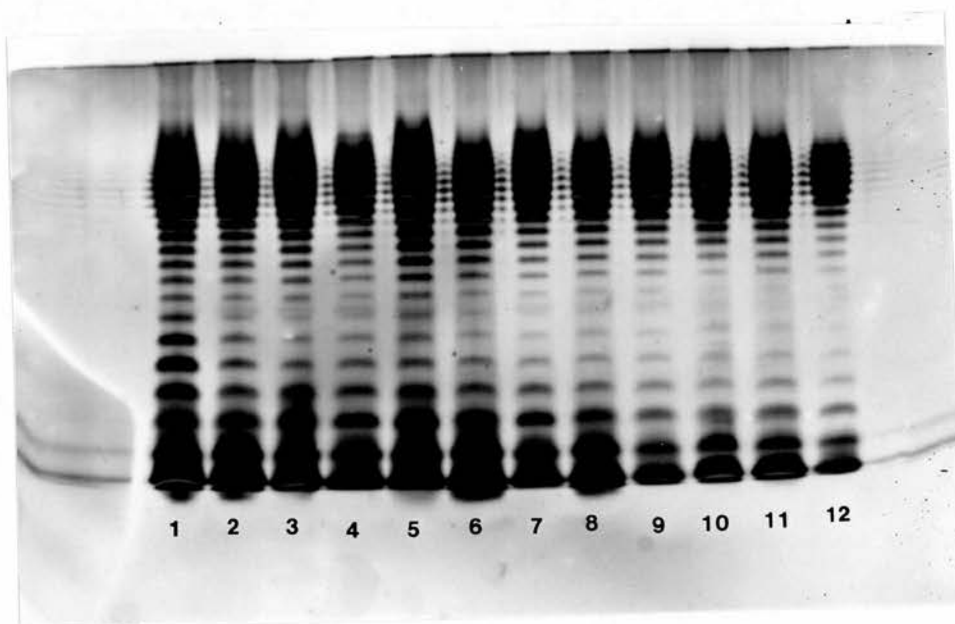


FIGURE 3:31. Silver stain of proteinase K digests from overnight cultures of E. coli O18:K$^-$ grown in media as described in Table 3:3. Growth media were: track 1 - medium 1; track 2 - medium 2; track 3 - medium 3; track 4 - medium 4; track 5 - medium 5; track 6 - medium 6; track 7 - medium 7; track 8 - medium 8; track 9 - medium 9; track 10 - medium 10; track 11 - medium 11; and track 12 - medium 12.

b) The same growth conditions were applied to an isogenic mutant of the above organism which produces little or no capsular material (E. coli O18:K$^-$). Once again, A_{525} was measured (table 3:3) and adjusted to 0.5 - 0.6 for proteinase K digestion followed by PAGE and silver staining for LPS (figure 3:31). Significant growth was obtained under all conditions (including PBS) as determined by measurement of optical density of bacterial suspensions. In figure 3:31, it can be seen that staining of high molecular weight lipopolysaccharide was very heavy, but little difference was obtained under different growth conditions. Growth of organisms in 100% serum and in dilutions of serum in PBS, produced heavier staining of medium molecular weight bands than under other conditions. Staining of these medium molecular weight bands became less prominant as serum was diluted in NB. Staining of low molecular weight components of LPS was fairly uniform in all media. Some media (100% serum, 50% serum:50% NB, 5% serum: 95% NB, and 100% NB) induced the production of a fast-migrating band as seen with E. coli O18:K1.

3:5:2. Alteration of LPS during Growth of E. coli O18:K$^-$.

An overnight culture in NB (5ml) was used to inoculate 100ml of NB. This was incubated as described previously. Samples were removed at 30min intervals for measurement of A_{525} against a nutrient broth blank. After measurement of optical density, bacteria were harvested, washed twice in PBS and resuspended to give an A_{525} of 0.5 to 0.6. Proteinase K digestion was then carried out, and samples were subjected to PAGE. The alteration in A_{525} was graphed versus time of sample after inoculation (figure 3:32), and shows that bacteria entered logarithmic phase of growth very rapidly, and

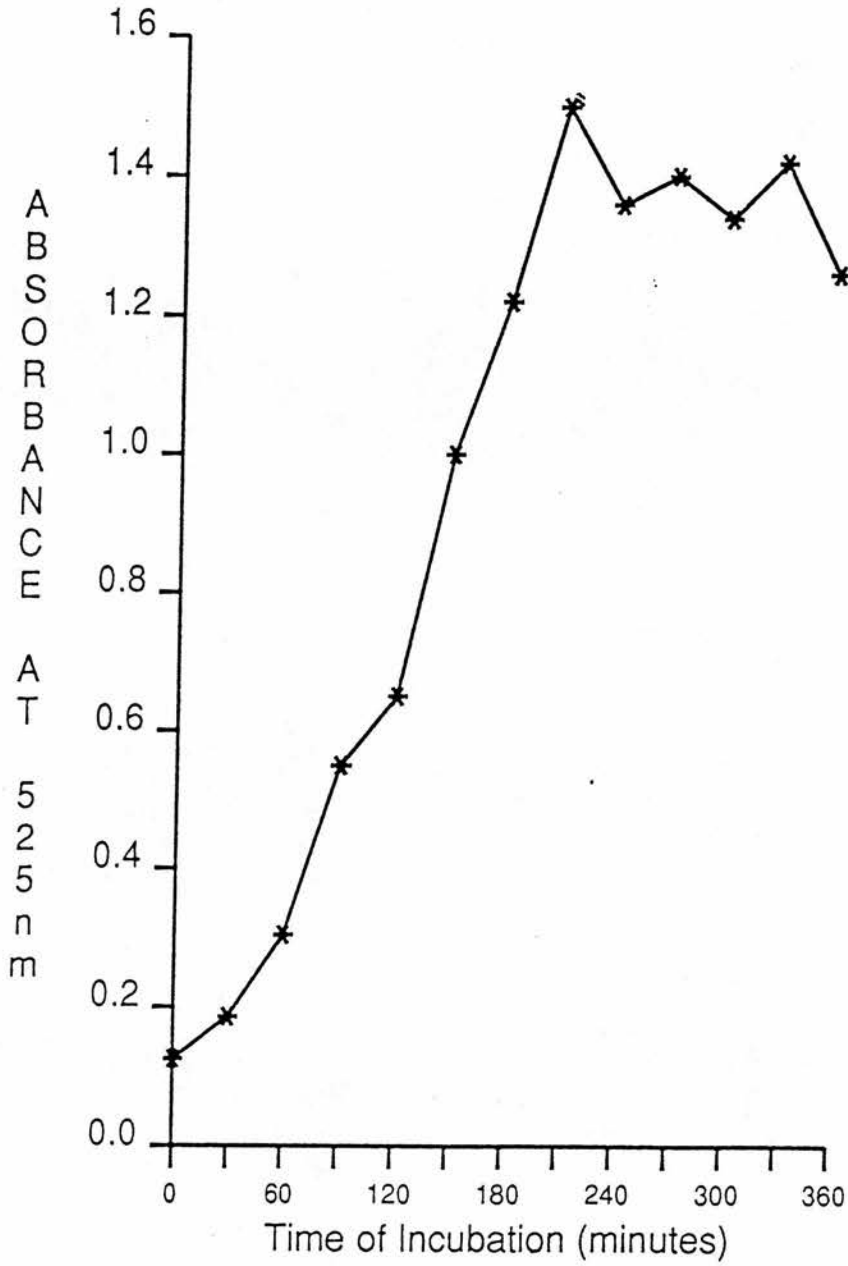


FIGURE 3:32. Growth of E. coli O18:K^- in Nutrient Broth as determined by measurement of absorbance of bacterial suspension at 525nm.

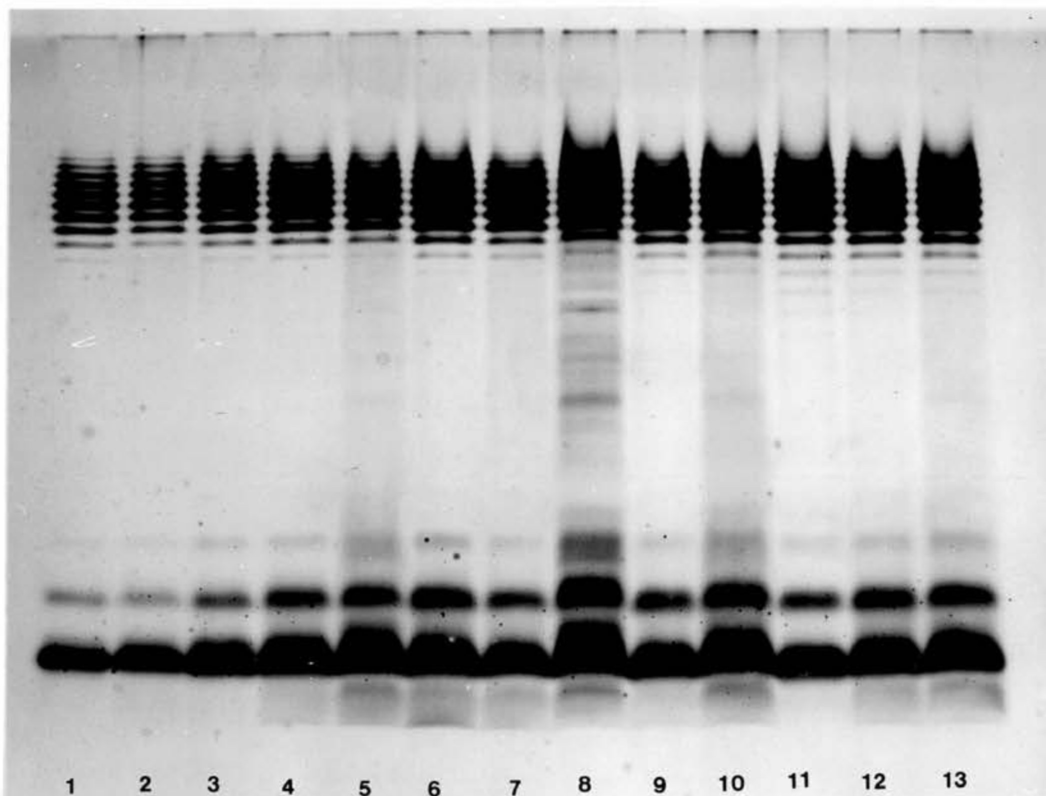


FIGURE 3:33. Silver stain of proteinase K digests of E. coli O18:K- grown in Nutrient Broth. Samples were removed at 30min intervals from 0min incubation (track 1) to 360min incubation (track 12). The corresponding growth curve is presented in Figure 3:32.

continued to proliferate rapidly until stationary phase was reached by 240 min after inoculation. Silver staining of PAG (figure 3:33) revealed that incomplete proteinase K digestion had occurred for samples removed at times 120, 210, and 240, but that LPS was not obscured. Increased staining of high molecular weight bands was observed over the course of growth. Increased staining of the first and second fast migrating bands occurred also with maximal staining occurring between 120 and 150 min. In addition, a third fast migrating band appeared behind these two at 60 min, and remained constant throughout after this point. Over and above these alterations, staining becomes apparent of material in front of the fastest migrating band after 90 min incubation - this may represent material observed previously.

3:5:3. Effect of Density of Bacterial Suspension on LPS Staining Intensity.

In view of the differences observed above between different LPS profles, a determination was made of the effect of density of bacterial suspension and volume of proteinase K digest loaded onto gel on the resultant LPS profile. For this, E. coli O18:K^- was cultured overnight. After harvesting and washing, the absorbance of the suspension was measured and adjusted to give three values between 0.50 and 0.65. The final A_{525} values obtained were 0.54, 0.58, and 0.64. Proteinase K extracts were prepared for each suspension, and two volumes (10ul and 20ul) of each were loaded onto PAG for electrophoresis. The results of silver staining (figure 3:34) show incomplete proteinase K digestion, but LPS profiles and staining density remained more or less constant in all samples.

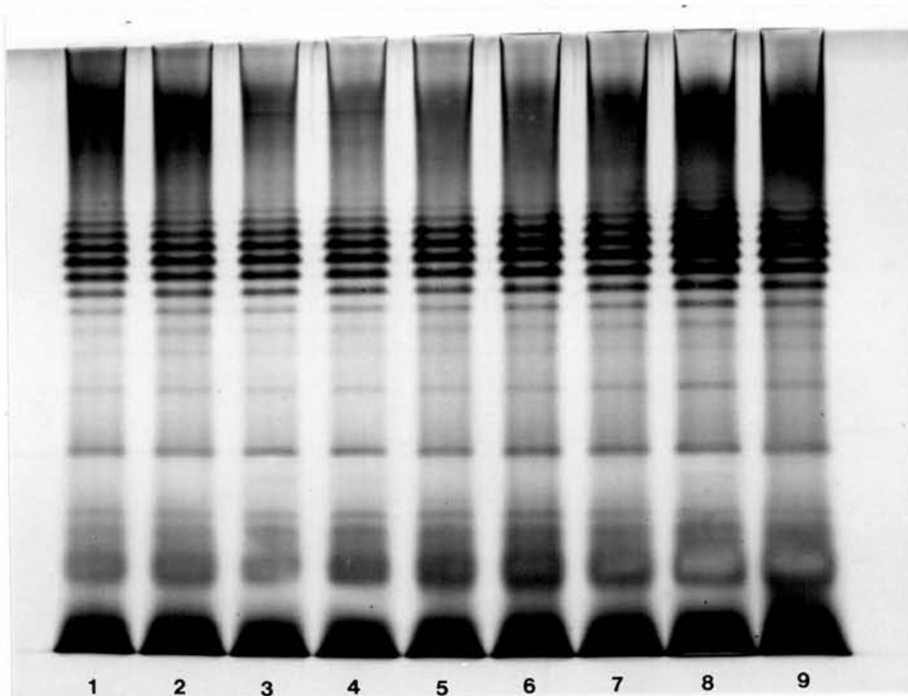


FIGURE 3:34. Silver stain of proteinase K digests of overnight cultures of E. coli O18:K-. Bacterial suspensions of different optical densities (A_{525} of 0.54 - tracks 1, 2, and 7; A_{525} of 0.58 - tracks 3, 4, and 8; and A_{525} of 0.64 - tracks 5, 6, and 9) and different volumes of proteinase K digest were loaded (10ul in tracks 1 to 6 and 20ul in tracks 7 to 9).

3:5:4. Growth of E. coli under Different Nutrient Conditions.

Growth curves were carried out for *E. coli* O18:K⁻ cultured in four media to assess growth phase of cells and thus relevant time points for analysis of LPS. Figure 3:35 shows the results obtained for growth in NB, MALKA (minimal medium), NDM (nitrogen deficient medium), and HSS (heat-inactivated sheep serum). The lag phases in NB and NDM were very short, and A_{525} had doubled within 60min. Bacterial growth in NB continued for 180min, after which point growth slowed down and stationary phase was reached by 300min after inoculation. In NDM growth continued at a rapid rate for 240min, at which point proliferation ceased abruptly. The lag phases for both MALKA and HSS were longer. When grown in HSS log phase was entered by 90min, and continued until 210min, at which point growth a slower rate of growth was apparent. Stationary phase was apparently entered by 270min. MALKA resulted in entry into logarithmic growth only after 120min incubation. Multiplication of bacteria continued rapidly until 360min, at which point growth ceased abruptly.

3:5:5. Growth of E. coli under Magnesium Limitation.

Modifications of MALKA containing 10% and 1% of the concentration of magnesium (M10 and M01 respectively) were used as growth media for *E. coli* O18:K⁻. Samples were removed every 30min for determination of A_{525}. Results of A_{525} were graphed versus time for both M10 and M01 and were compared to the previously obtained growth curve for unmodified MALKA. Figure 3:36 indicates that a lag phase of approximately 150min was present under both nutrient conditions. After this point, bacterial multiplication occured, but at a far

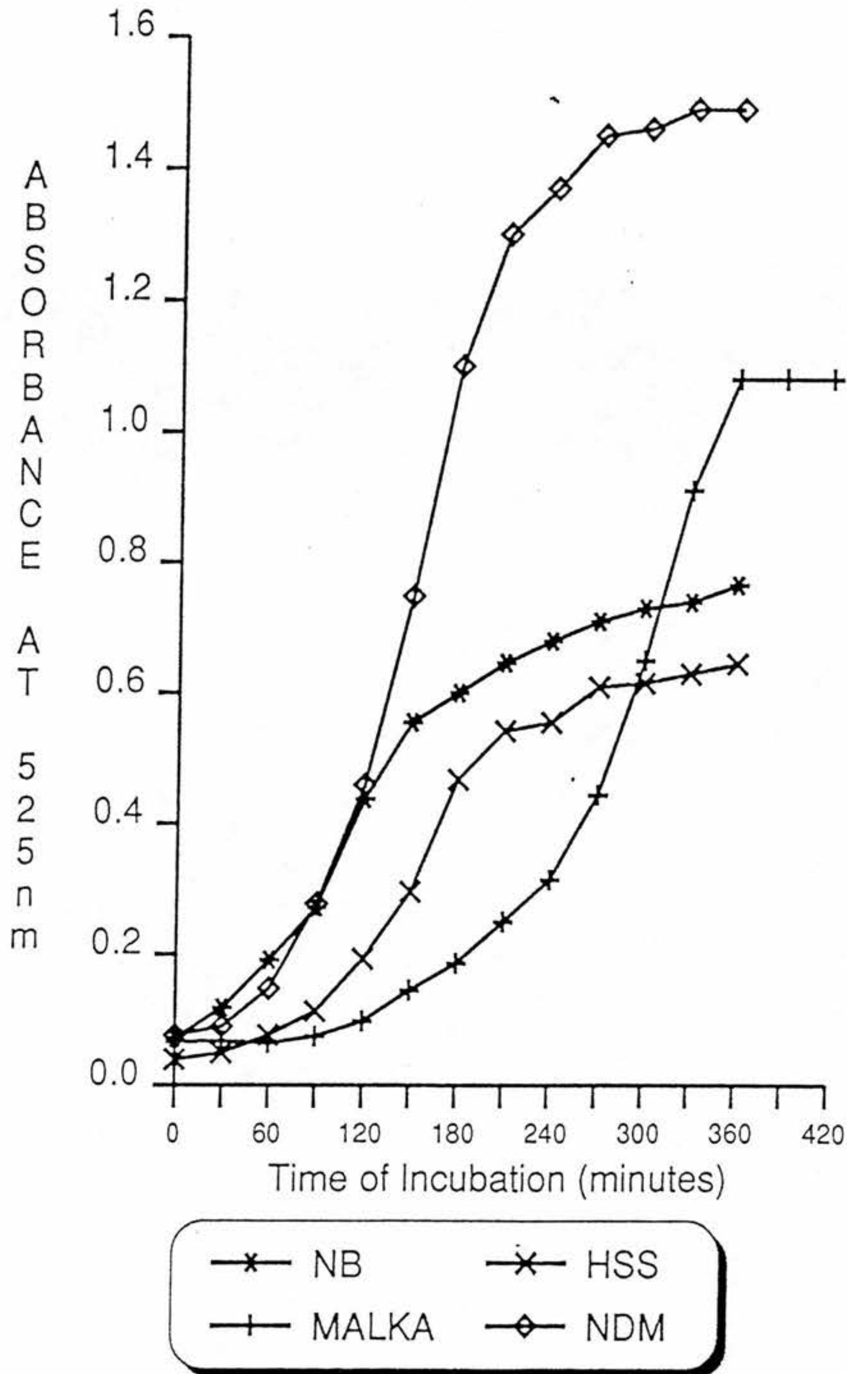


FIGURE 3:35. Growth of E. coli O18:K^{-} in four media - NB, MALKA, HSS, and NDM (as detailed in the text) as determined by measurement of absorbance of bacterial suspension at 525nm.

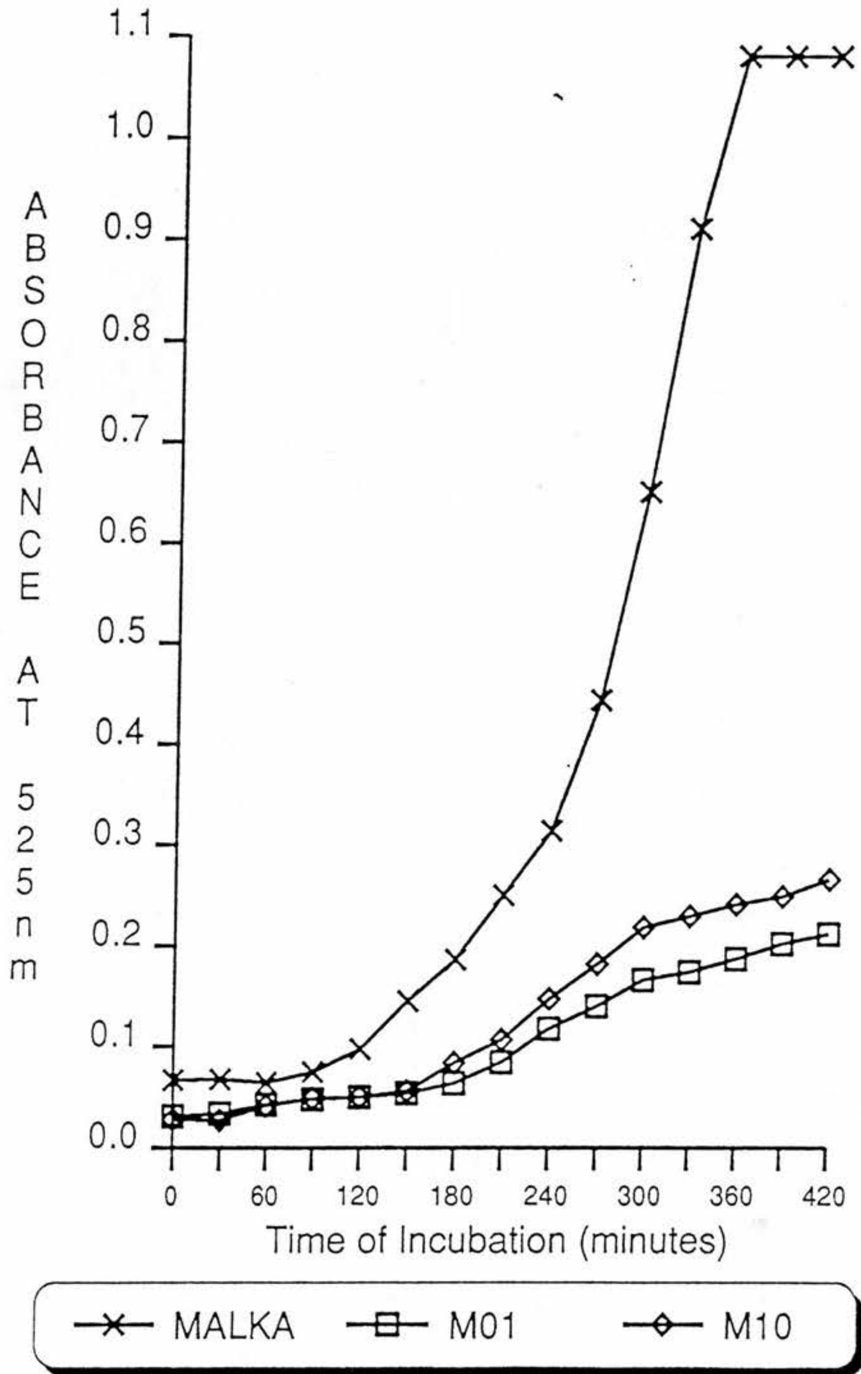


FIGURE 3:36. Growth of E. coli O18:K^{-} in three minimal media (as detailed in text) as determined by measurement measurement of absorbance of bacterial suspension at 525nm.

slower rate than for any other medium. Growth continued at these rates until 300min, from which time a slower rate of growth continued until 450min. After overnight incubation (24h) the A_{525} readings for M10 and M01 were 1.45 and 1.8 respectively.

3:5:6. Effect of Growth Medium and Growth Phase on LPS Expression and Binding of Monoclonal Antibodies to Core and O-antigen.

In view of the above results it was decided that hourly samples would be sufficient to cover all phases of growth for proteinase K extraction to visualise LPS, and flow cytometry analysis to determine binding of monoclonal antibodies to core and O-antigen.

An overnight culture of *E. coli* O18:K^- was harvested and washed. Bacteria were then resuspended to their original volume in PBS. After measurement of A_{525} (1.45) 5ml of suspension was inoculated into each of three media (NB, MALKA, and HSS). Samples were removed hourly for determination of A_{525}, for preparation of proteinase K extracts, and for incubation with monoclonal antibodies.

Figure 3:37 indicates that the growth curves produced in these media are similar to those obtained previously (figure 3:35).

LPS profiles of bacteria grown in NB is also similar to that obtained previously. Both NB and MALKA (figures 3:38a & b) show heavy staining of large bands of components running at the front, while heat-inactivated serum (figure 3:38c) produced a smaller more discrete band. Little alteration in LPS appeared to occur in bacteria cultured in MALKA, but in NB and HSS heavier staining appeared in later samples. Growth in serum induced rapid formation of a very high molecular weight component which was not observed in other samples including inoculum. The expression of this component,

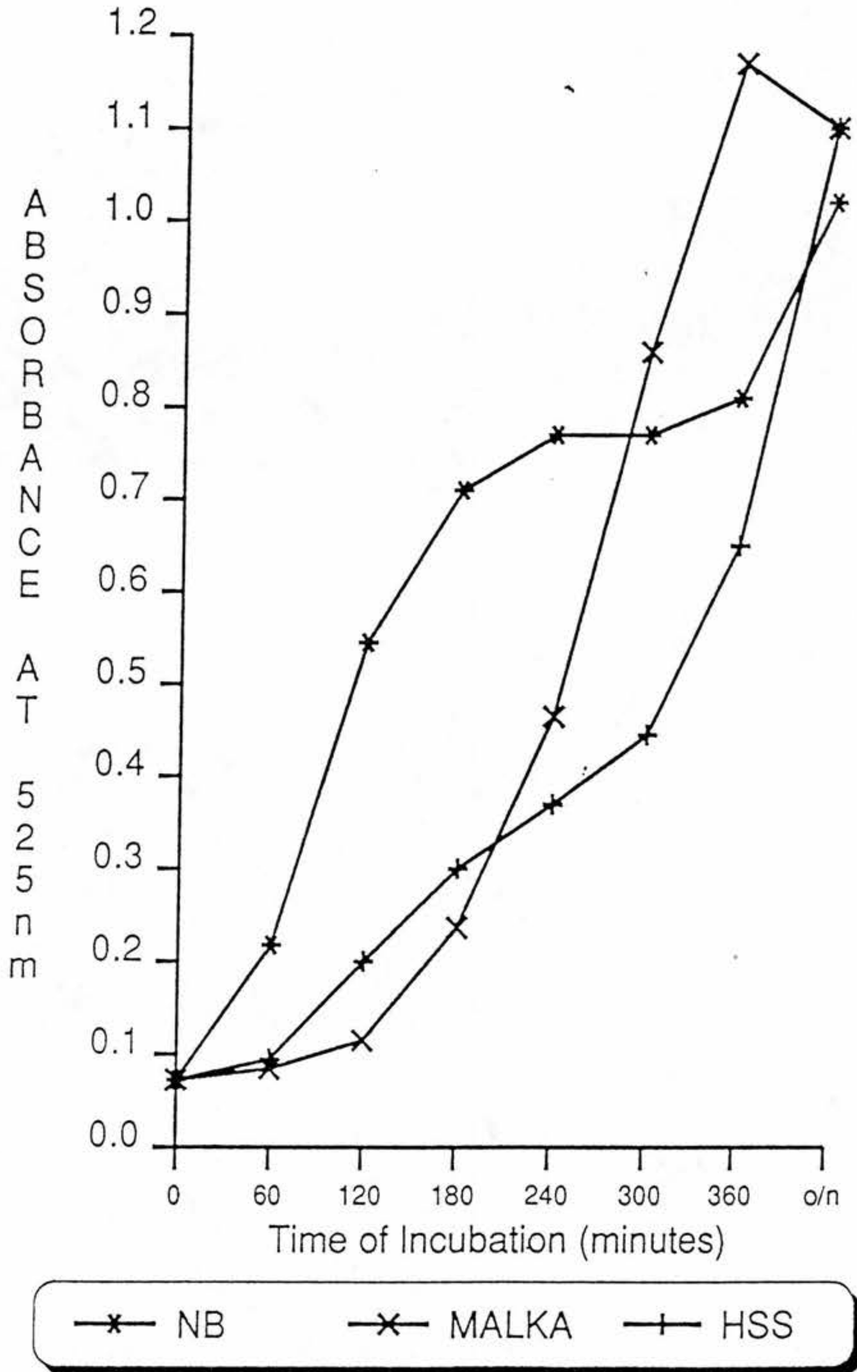
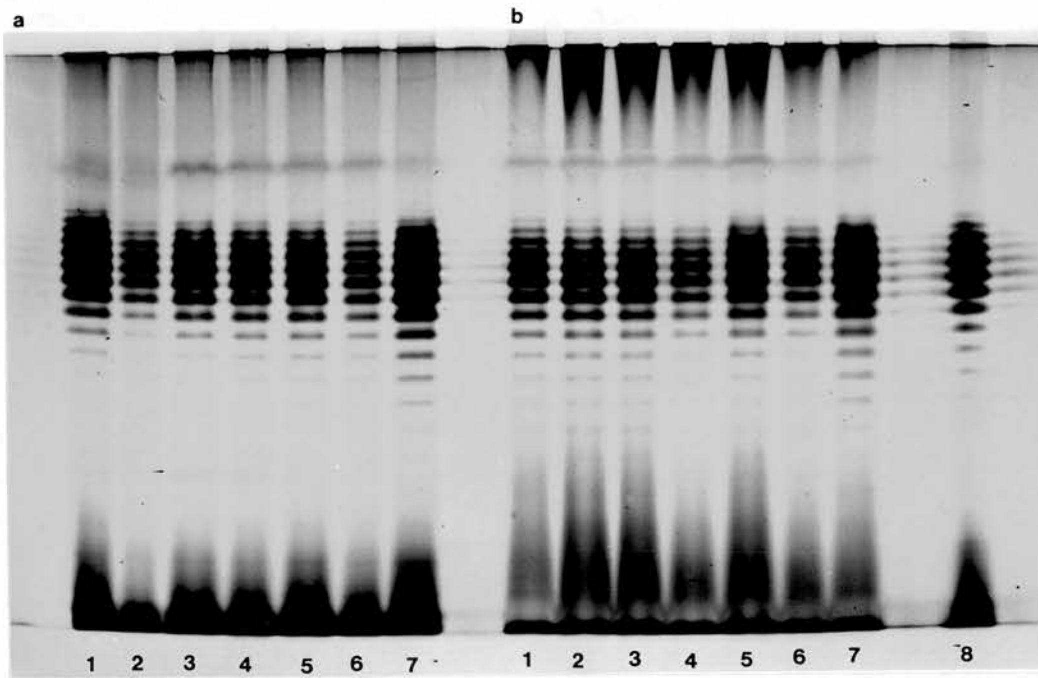


FIGURE 3:37. Growth of E. coli O18:K⁻ in three media (see text for details) as determined by measurement of absorbance of bacterial suspension at 525nm.



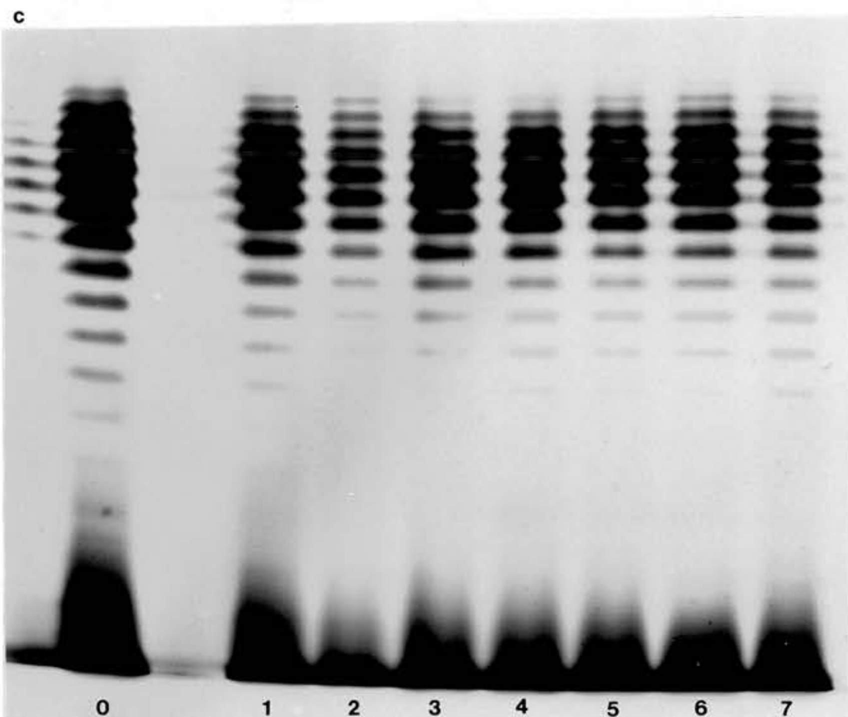


FIGURE 3:38. Silver stain of proteinase K digests (5ul) of E. coli O18:K- grown in nutrient broth (figure a), heat-inactivated sheep serum (figure b), and MALKA minimal medium (figure c). Samples were removed at 60min intervals from 60min incubation (track 1) to 360min incubation (track 6) plus a sample from overnight incubation. Inocula for (a) and (b) are represented in track 8 figure (b), and for (c) in track o of figure (c).

however, diminished as time progressed.

Analysis of the binding of monoclonal antibodies to core and O-antigen of this organism by flow cytometry produced the results presented in table 3:4 after subtraction of background fluorescence (bacteria incubated with only FITC-labelled anti-mouse IgG - see MATERIALS AND METHODS).

The results showed that growth in NB produced negligible binding of an anti-core monoclonal antibody with a mean of 1.0% of cells fluorescently labelled, while the anti-O-antigen monoclonal IgG showed high binding at all points of the growth curve (a mean of 54.1% of cells labelled). MALKA produced similar results to NB with means of -1.1% and 69.2% of bacteria labelled with core and O-antigen monoclonal antibodies respectively. Growth of bacteria in serum produced contrasting results because significant binding was obtained with both monoclonal antibodies during active growth (means of 33.9% and 47.4% with core and O-antigen monoclonal antibodies respectively). Two exceptions from this occurred at 60min after inoculation, and after overnight culture, where results were comparable to those obtained with NB and MALKA.

Overnight culture of this organism in NB was repeated, and results were similar to those obtained previously with -0.05% of cells binding core monoclonal and 48.28% of cells binding O-antigen specific monoclonal antibody (table 3:5).

Growth overnight in modified MALKA media containing 10% and 1% of the magnesium concentration (M10 and M01 respectively) produced results similar to those obtained for overnight growth in unmodified MALKA (table 3:5).

TABLE 3:4. Percentage of E. coli O18:K⁻ Binding Monoclonal Antibodies to Core and O-antigen over Growth Curves under Different Nutrient Conditions.

TIME minutes)	Percent of *E. coli* binding Monoclonal Antibody in:					
	Nutrient Broth		MALKA		Serum	
	core[1]	O-antigen[2]	core	O-antigen	core	O-antigen
0	0.01	43.76	0.01	43.76	0.01	43.76
60	6.16	57.84	0.58	51.19	4.65	29.02
120	2.09	49.23	-11.81	29.69	40.30	38.85
180	-0.84	35.50	0.52	43.51	56.87	58.16
240	0.06	80.37	0.48	23.78	28.06	43.00
300	0.20	80.53	0.83	86.98	24.77	50.53
360	0.07	69.79	-4.18	77.75	19.48	46.66
o/n	0.38	15.53	0.94	65.30	2.89	23.08

1. McAb SZ27/150.3, anti-core.
2. McAb SZ184/2.5.5, anti-O18 Oantigen.

TABLE 3:5. Percentage of E. coli O18:K⁻ Binding Monoclonal Antibodies to Core and O-antigen after Overnight Growth in Magnesium Limiting Media.

MEDIUM	PERCENTAGE *E. coli* BINDING TO:	
	CORE[1]	O-ANTIGEN[2]
NB	-0.14	48.24
M10	2.68	61.60
M01	-0.05	64.14

1. as above.
2. as above.

3:5:7. Comparison of Capsulate and Non-capsulate E. coli 018 Grown in Untreated and Heat-inactivated Sheep Serum.

E. coli 018:K1 and 018:K$^-$ were prepared as described previously, and optical densities of suspensions were measured (K1 = 1.18, and K$^-$ = 1.10). Two 5ml volumes of each suspension were inoculated into 100ml of sheep serum (SS) and heat-inactivated sheep serum (HSS) from the same source, and subjected to the culture conditions mentioned above. Samples were removed every 60 min for determination of A_{525}, proteinase K digestion followed by PAGE, and analysis of binding of antibodies to core and O-antigen epitopes by flow cytometry.

The growth curves of the two organisms under each condition (figure 3:39) showed that both organisms divided more rapidly in HSS than in SS. Both growth curves and final optical density readings for organisms grown in HSS were similar to those obtained previously under the same conditions. Optical densities of both K1 and K$^-$ grown in HSS were within 0.05 units at all points.

When grown in untreated serum E. coli 018:K1 grew at a steady slow rate throughout, with A_{525} at 360min of approximately 60% of that in HSS. In contrast, 018:K$^-$ shows only a small increase on cell number followed by a decline. From 240min onwards very rapid cell division was observed, matching the growth rate in HSS.

The lipopolysaccharide profiles (figure 3:40a-d) show that in untreated serum both organisms produced increasing staining of core and O-antigen bands of LPS with time. In HSS increased staining of O-antigen was noted to occur, but this was more pronounced in E. coli 018:K1. Staining of core components of bacteria grown in HSS did not change markedly from the inoculum.

The binding of monoclonal antibodies was assessed as above. The

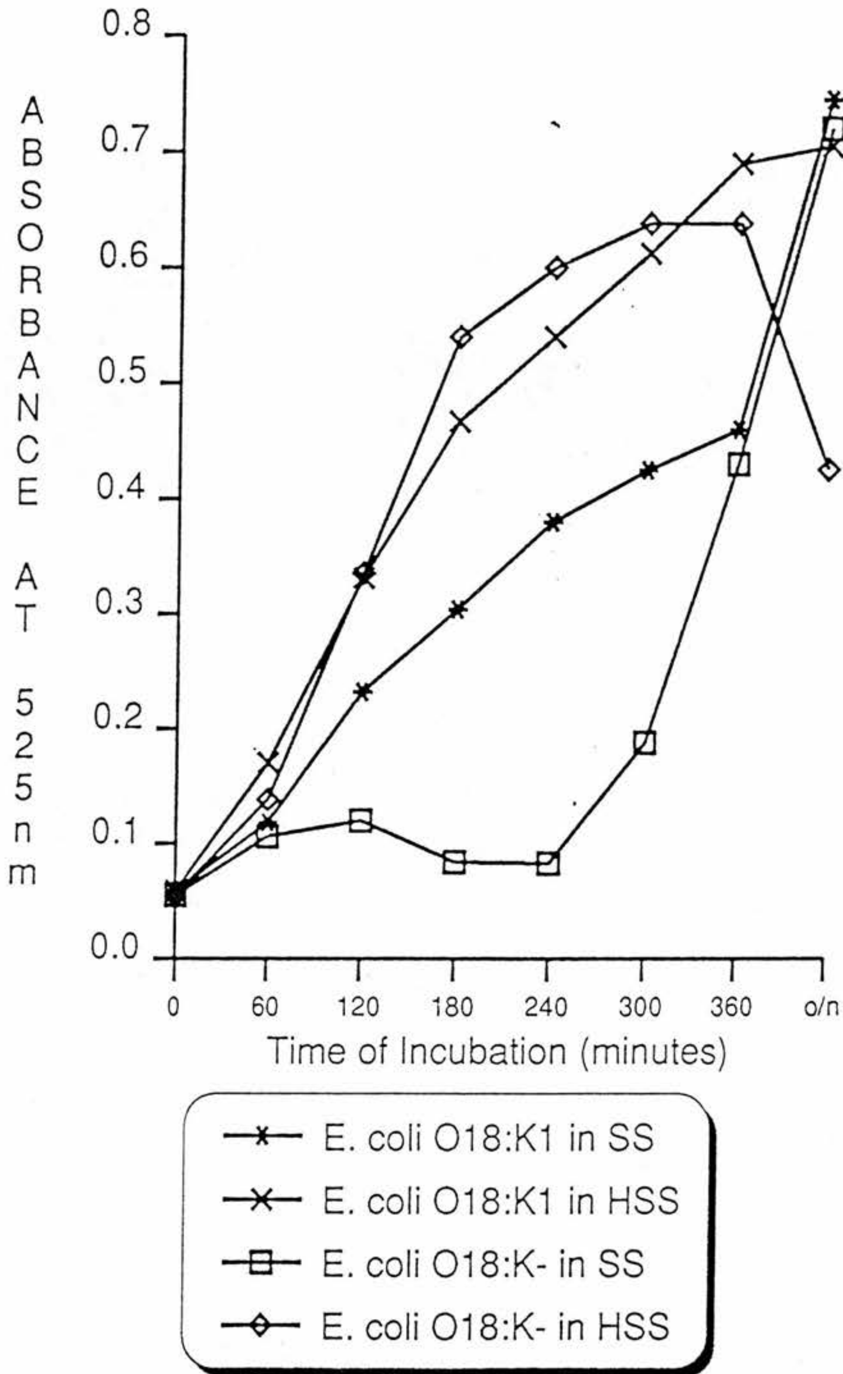


FIGURE 3:39. Growth of E. coli O18:K$^-$ and E. coli O18:K1 in untreated sheep serum (SS) and heat-inactivated sheep serum (HSS) as determined by measurement of absorbance of bacterial suspension at 525nm.

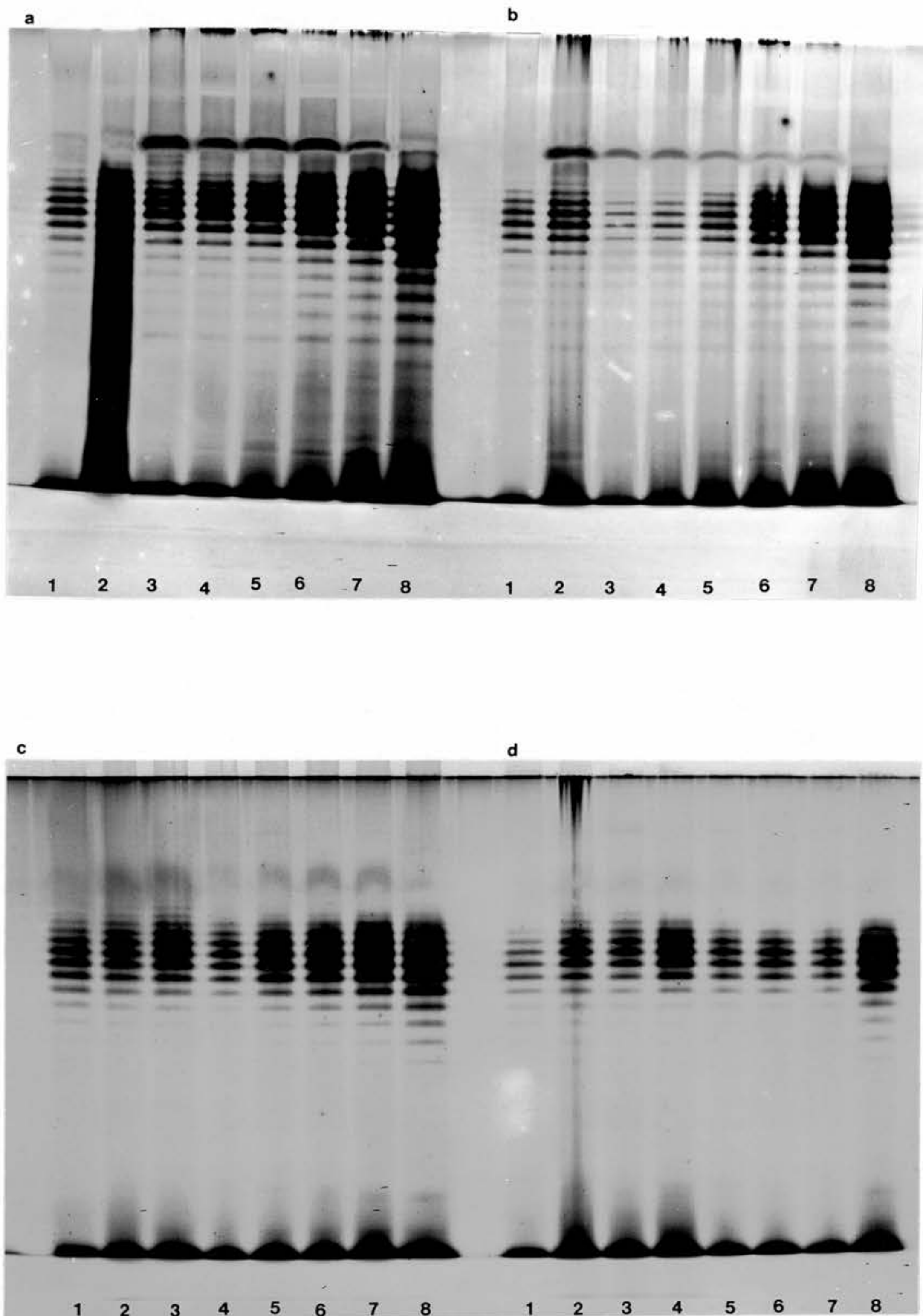


FIGURE 3:40. Silver stain of proteinase K digests of E. coli O18:K1 (figures a & c) and E. coli O18:K$^-$ (figures b & d). Bacteria were cultured in untreated sheep serum (figures a & b) and in heat-inactivated sheep serum (figures c & d). The inocula for each is shown in track 1. Samples were removed at 60min intervals from 60min incubation (track 2) to 360min incubation (track 7) plus a sample from overnight incubation.

TABLE 3:6. Percentage of E. coli O18:K⁻ and O18:K1 Binding Monoclonal Antibodies to Core and O-antigen over Growth Curves in Untreated and Heat-inactivated Serum.

Time (minutes)	Percentage of bacteria binding Monoclonal Antibody after growth in:							
	E. coli O18:K⁻				*E. coli* O18:K1			
	Untreated Serum		Heated Serum		Untreated Serum		Heated Serum	
	core[1]	O-antigen[2]	core	O-antigen	core	O-antigen	core	O-antigen
0	2.01	90.17	2.01	90.17	0.45	60.46	0.45	60.46
60	12.75	-20.91	-35.81	-34.24	13.78	-7.77	-0.63	21.48
120	41.81	22.81	26.83	39.18	27.49	34.49	4.79	53.64
180	25.74	40.25	26.48	48.84	17.86	45.33	-9.58	20.46
240	36.52	48.59	11.95	50.76	-3.55	-3.93	-5.23	39.41
300	34.45	28.23	-0.26	35.39	-3.08	1.89	-12.11	23.87
360	25.86	61.40	-11.52	29.34	8.21	11.60	-2.08	19.23
o/n	18.45	73.23	16.78	43.80	11.17	15.30	3.84	15.22

1 and 2: monoclonal antibodies as before.

results presented in table 3:6 showed one or two anomalous points, but overall they indicated once again that E. coli when grown in HSS permitted access of a core-specific monoclonal antibody as well as an O-antigen specific monoclonal antibody. Similar binding characteristics were also obtained with this organism grown in untreated serum. E. coli O18:K1 grown in SS showed much lower binding to both core and O-antigen, although from times 60min to 180min limited binding was observed by the anti-core monoclonal antibody. After this point, binding to both core and O-antigen was very low. In HSS binding to core generally produced negative values (i.e lower than control), but binding to O-antigen occurred for a mean of 29.7% of bacteria cultured for between 60min and 360min.

3:5:8. Growth of E. coli in Absorbed and Non-Absorbed Serum, and Effect on Binding of Monoclonal Antibodies to Core and O-antigen.

Absorption of serum was carried out with organisms of identical and unrelated O-antigen structures (E. coli O18:K^- and E. coli O86:K61 respectively) to that of the bacterium under analysis. Washed suspensions of each organism were prepared in PBS at a concentration of 10^{10}cells/ml. A volume (1.0ml) of each was placed in clean sterile Universal bottles and bacteria were pelleted by centrifugation. After removal of supernate, bacteria were resuspended in 10ml of untreated sheep serum, giving a final concentration of 10^9cells/ml. The suspension was immediately centrifuged then serum was decanted and used to resuspend a fresh bacterial pellet. Suspensions were immediately centrifuged as this was determined previously to effectively remove LPS-specific antibodies as shown in an ELISA system (section 3:4). Centrifugation

TABLE 3:7. Percentage of *E. coli* O18:K⁻ and O18:K1 Binding Monoclonal Antibodies to Core and O-antigen in Absorbed and Non-absorbed Serum.

Absorbant	Time	Percent of bacteria binding Monoclonal antibody to:			
		E. coli O18:K⁻		*E. coli* O18:K1	
		core[1]	O-antigen[2]	core	O-antigen
nil	2h	5.27	45.57	3.16	61.48
	4h	-15.81	26.46	0.02	40.58
	6h	-5.71	30.92	-0.90	42.67
E. coli O18K⁻	2h	-3.10	27.81	2.07	6.77
	4h	-1.50	21.33	-34.51	-30.37
	6h	nd*	19.84	-1.56	3.22
E. coli O86:K61	2h	-8.19	35.51	-0.48	4.08
	4h	-7.08	17.42	-0.04	1.68
	6h	-0.26	34.10	-0.84	1.69

1 and 2: monoclonal antibodies as before.

*: nd, not done.

was again carried out and the serum was used once more for absorption. The absorbed serum (ASS) was stored at 4°C overnight until required.

Inocula for growth in SS and ASS were prepared as previously described, and 0.2ml of suspension was inoculated into 4.0ml of each serum preparation: a) untreated serum; b) serum absorbed with O18:K^- cells; and c) serum absorbed with O86:K61 cells. After inoculation, bacteria were incubated at 37°C and 1.0ml samples were removed every 120min. Once washed, bacteria were reacted with monoclonal antibodies followed by fluorescein-labelled anti-mouse IgG at a dilution of 1:80.

Table 3:7 indicates the percentage of bacteria binding monoclonal antibodies for each growth condition. Many values were below those obtained in control samples reacted with only secondary antibody, although most of these were less than 5.0% below control. Three results were obtained with percentage values of 15.0% or more below controls. *E. coli* O18:K^- showed very low binding by anti-core monoclonal antibody in contrast to previous results, but binding to O-antigen occurred at reasonably high levels in absorbed and non-absorbed sera. Culture of *E. coli* O18:K1 in non-absorbed serum produced similar results to those obtained previously with anti-O-antigen monoclonal antibody, but binding with core monoclonal antibody was negligible. Growth of the capsulate strain (O18:K1) in serum absorbed with either O-serotype of bacterium resulted in negligible binding to core (as obtained in non-absorbed serum) and only very low binding by monoclonal antibody to O-antigen.

3:6. Activation of Limulus Amoebocyte Lysate by Lipopolysaccharide and Inhibition with Immunoglobulins.

3:6:1. Measurement of Limulus Amoebocyte Lysate Activity of Purified Lipopolysaccharides.

Comparisons were made of the capacity of purified LPS from several genera of bacteria to activate *Limulus* amoebocyte lysate in a chromogenic assay as described in MATERIALS AND METHODS. The assay was repeated several times for the measurement of endotoxins.

a) Lipopolysaccharides from one smooth and one rough organism (*E. coli* O18 and *S. typhimurium* R878 Rc respectively) were assayed. A 5-fold dilution series of each LPS was prepared in sterile pyrogen-free distilled water from a concentration of 10ng/ml downwards. Results were graphed as shown in figure 3:41a. At the highest concentration, both LPS showed comparable activities. The activity of R878 LPS fell rapidly from this point to give a minimal reactivity with LAL at 3.2×10^{-3}ng/ml. Endotoxin from *E. coli* O18 retained activity at 2.0ng/ml, but activity fell rapidly to a minimal value at 1.6×10^{-2}ng/ml.

b) A comparison was made between a variety of S-LPS from *E. coli* (O18, O16, and O6) and from *P. aeruginosa* Habs type 1 (figure 3:41b). A similar activity curve to that above was obtained with O18 LPS with a plateau at high concentrations and a baseline at approximately 3.2×10^{-3}ng/ml LPS. Similar curves were obtained with all *E. coli* LPS, but LPS from *P. aeruginosa* Habs type 1 showed a lower activity at all points, with minimal activity occurring between 1.6×10^{-2} and 8.0×10^{-2}ng/ml LPS.

c) Rough LPS from three mutants of *S. typhimurium* were assayed in

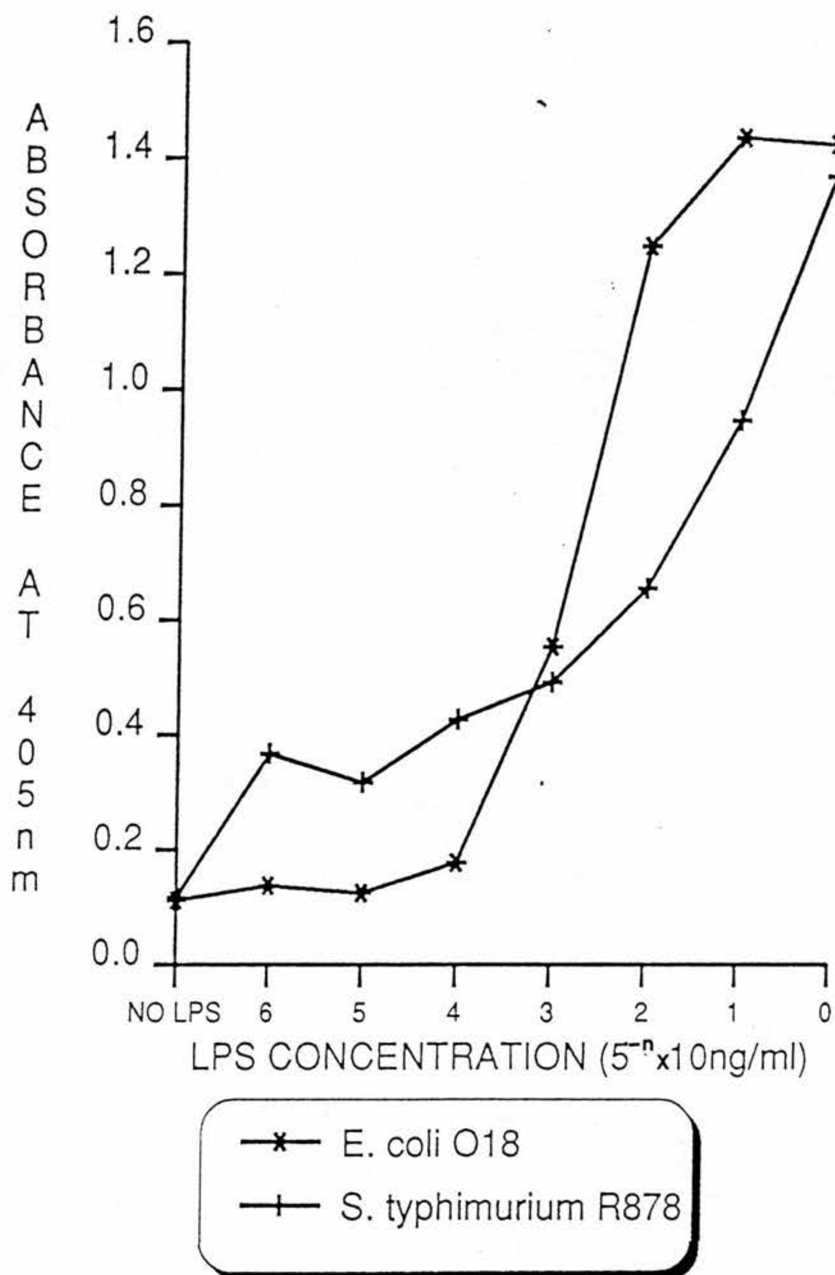


FIGURE 3:41a. Activity of a smooth LPS (from E. coli O18) and a rough LPS (S. typhimurium R878, Rc) in a chromogenic Limulus amoebocyte lysate (LAL) assay.

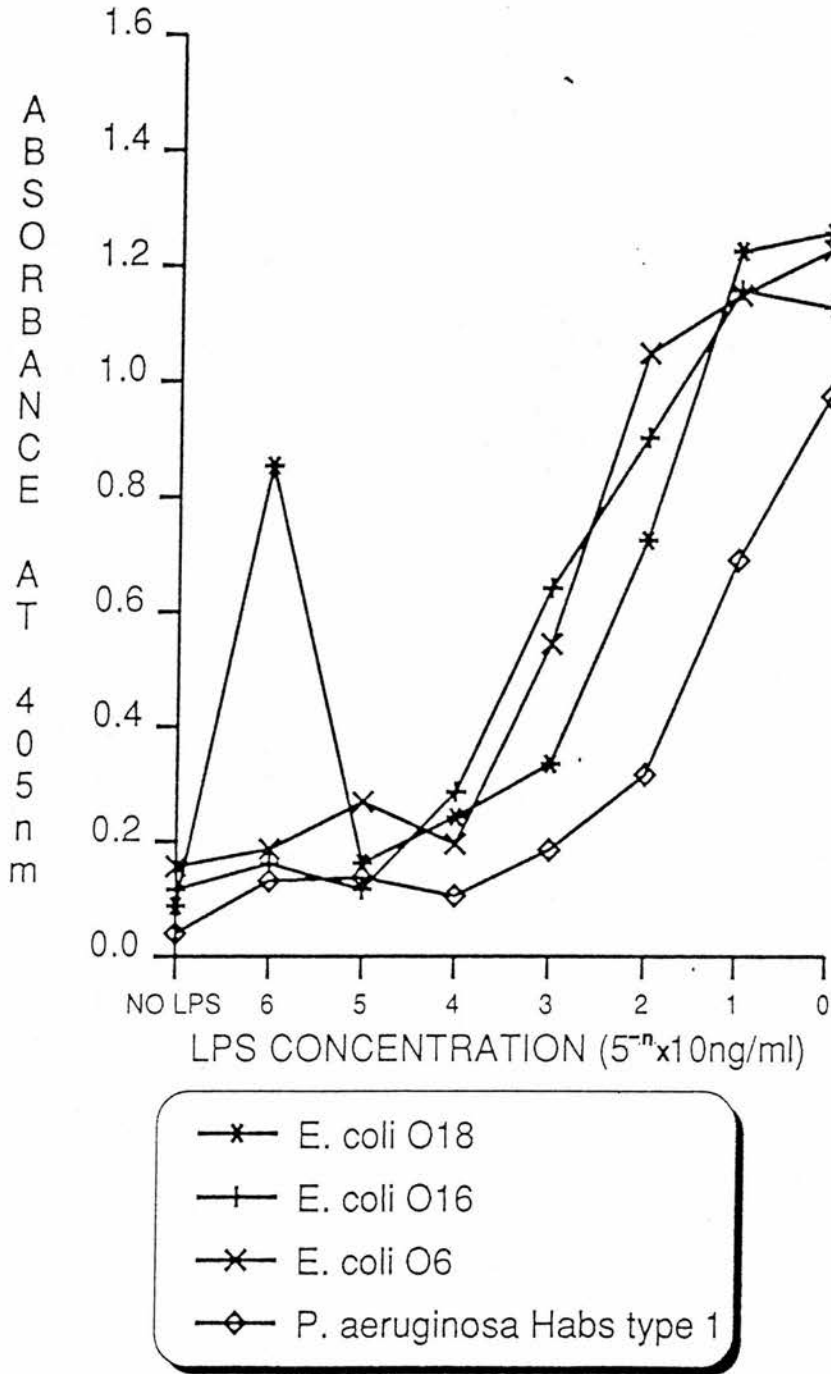


FIGURE 3:41b. Activity of four smooth lipopolysaccharides (as described in the legend) in a chromogenic LAL assay.

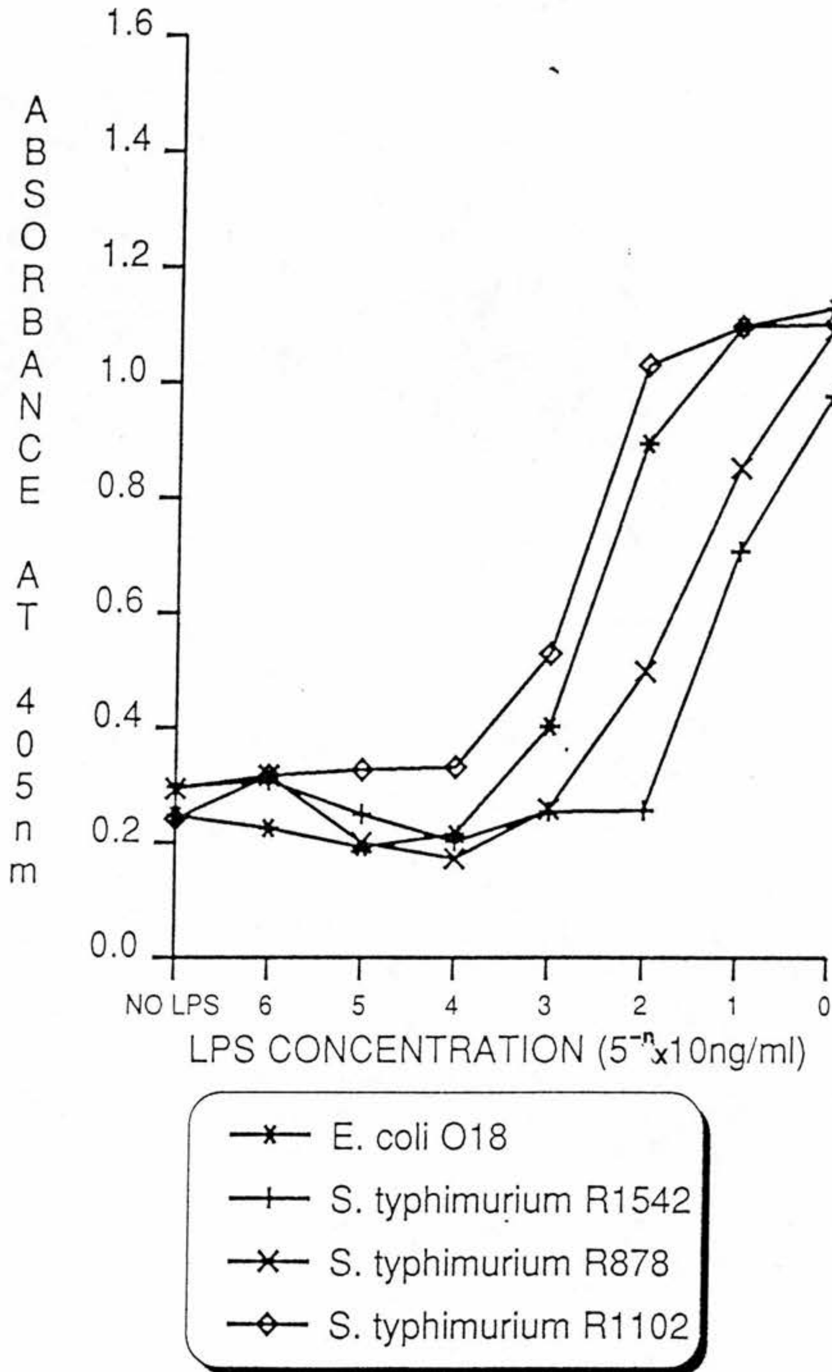


FIGURE 3:41c. Activity of three rough <u>S. typhimurium</u> lipopolysaccharides (as detailed in legend) in comprison to <u>E. coli</u> O18 LPS in a chromogenic LAL assay.

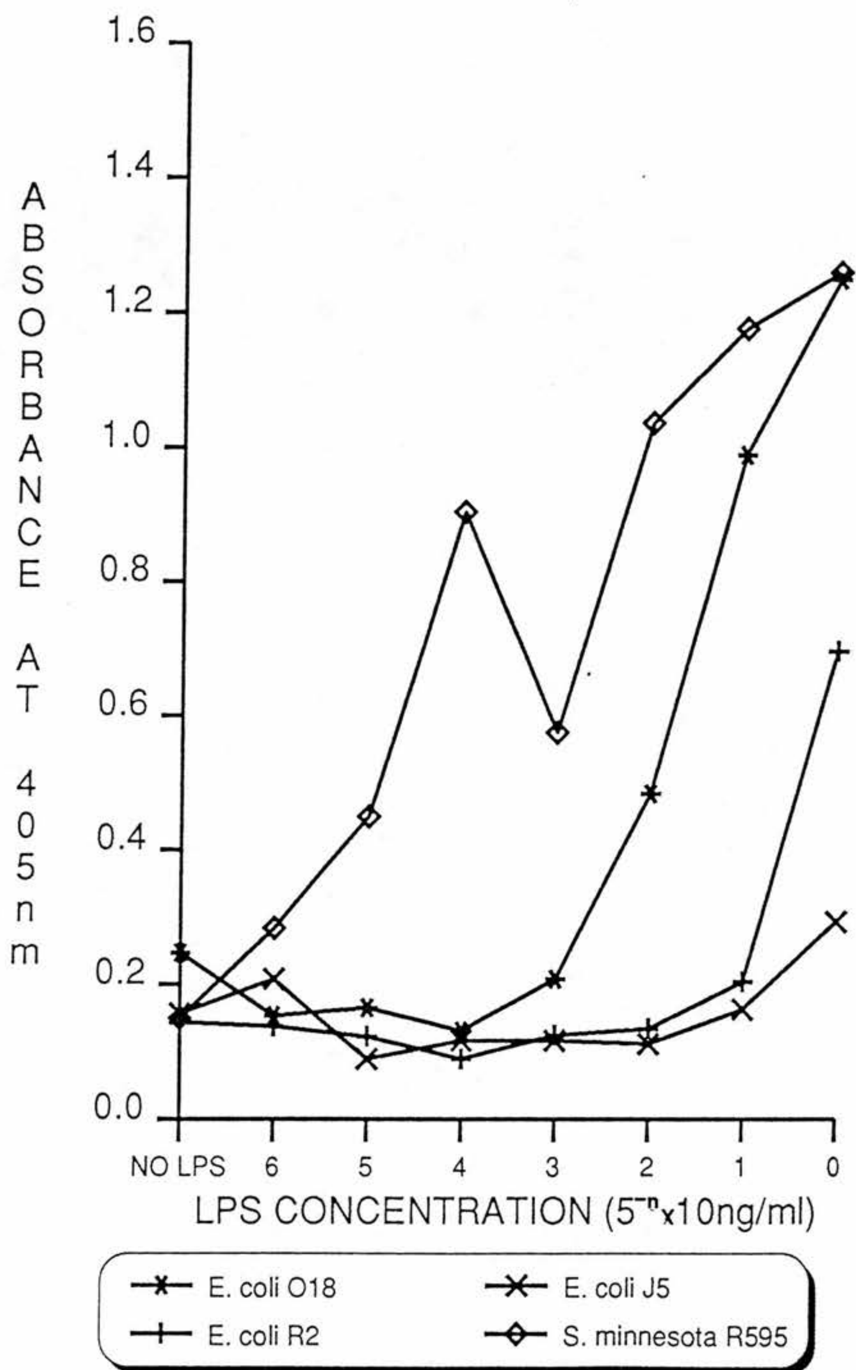


FIGURE 3:41d. Activity of three rough LPS (as described in the legend) and one smooth LPS (E. coli O18) in a chromogenic LAL assay.

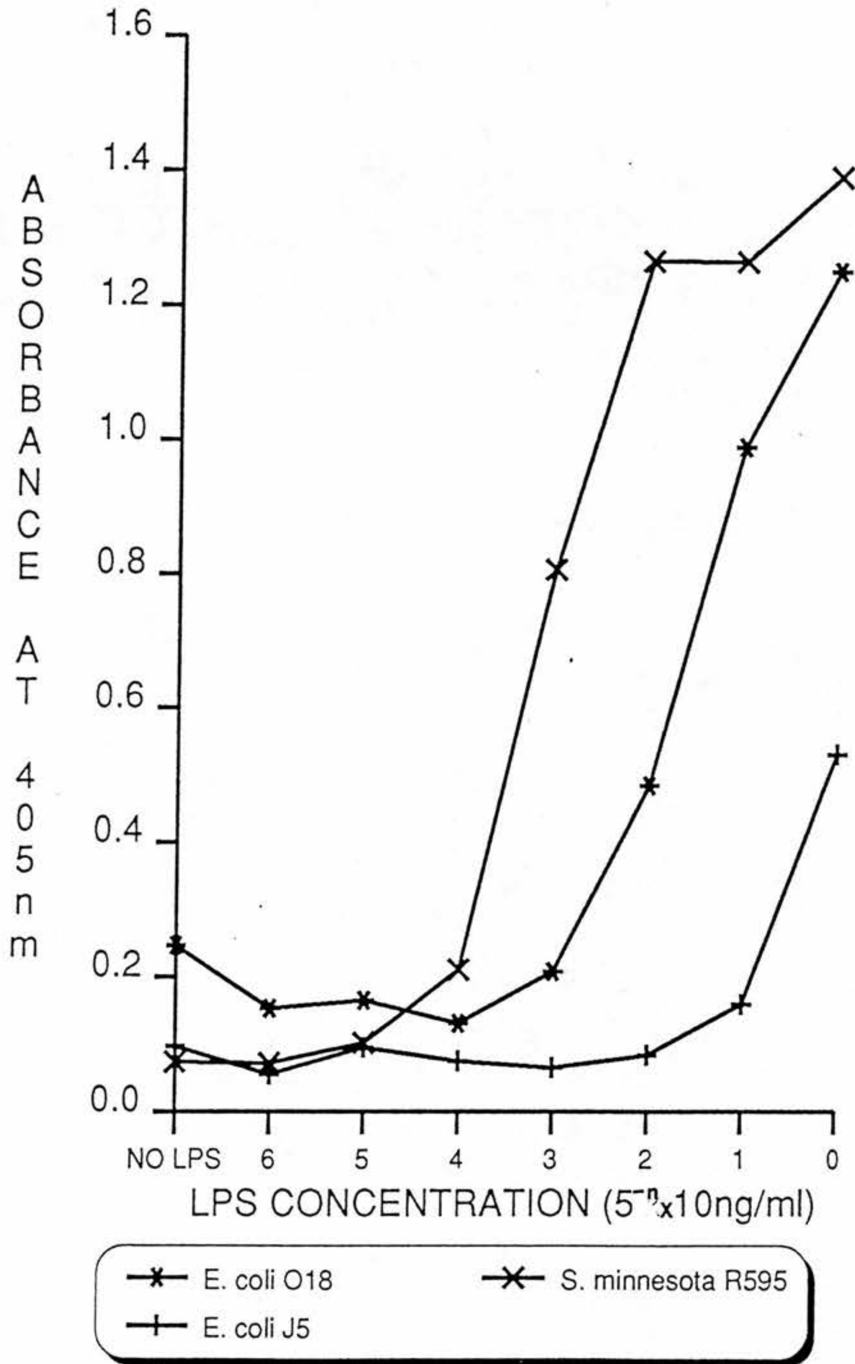


FIGURE 3:41e. Activity of S. minnesota R595 LPS and E. coli J5 LPS in a chromogenic LAL assay in comparison to E. coli O18 LPS.

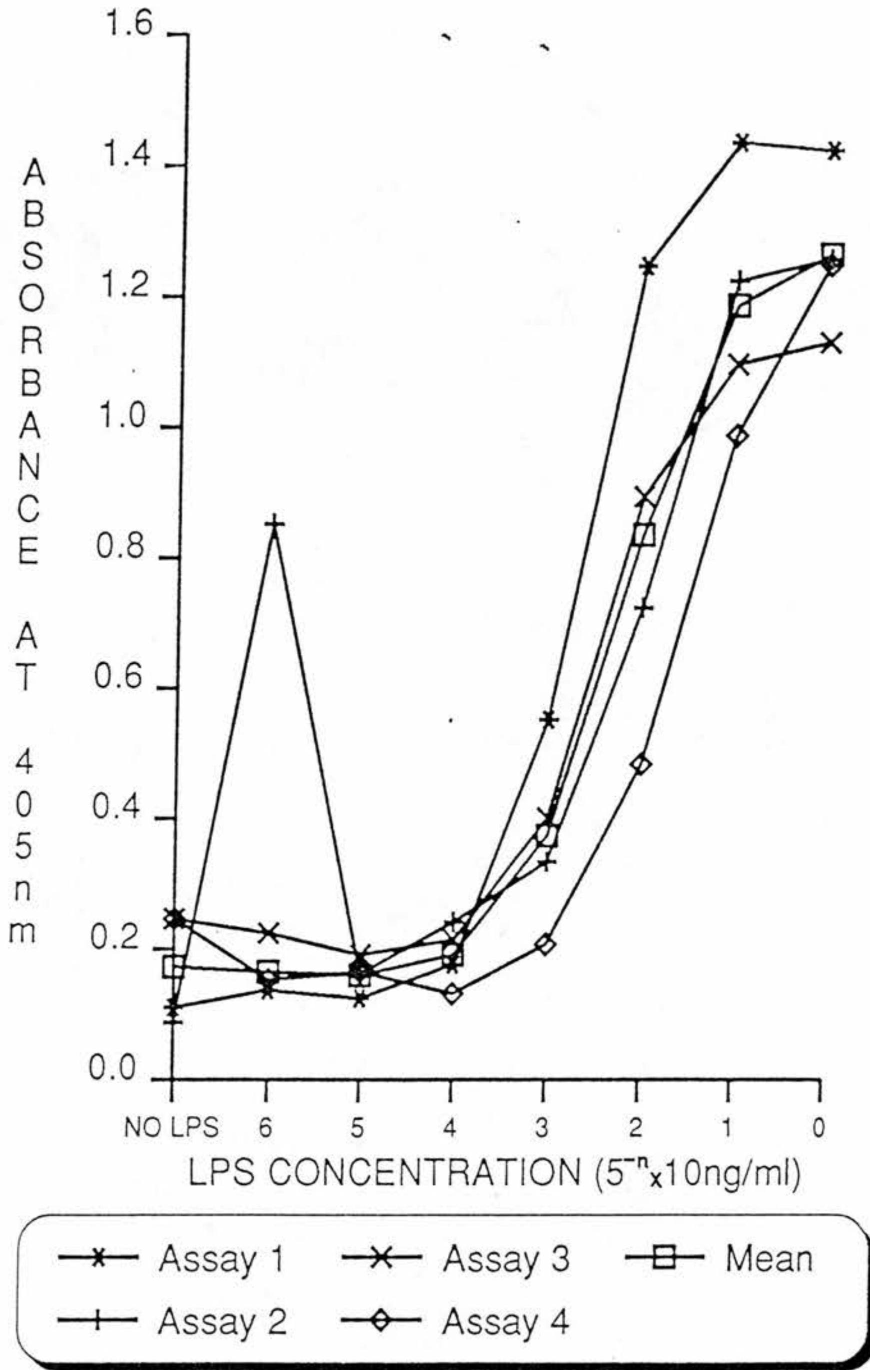


FIGURE 3:42. Activity of E. coli O18 LPS in repeated LAL assays (see figure 3:41a-e) plus a curve representing the mean values of all assays.

LAL and compared with O18 LPS. Figure 3:41c indicates that R1542 (Ra) LPS possessed low activity at high LPS concentrations and that activity fell sharply to a baseline value at 4.0×10^{-1}ng/ml. Endotoxin from R878 (Rc) had a similar activity to O18 LPS at 10.0nn/ml, but activity dropped sharply to a minimal value at $1.6-8.0 \times 10^{-2}$ng/ml. Rough LPS from R1102 (Re) showed activity almost identical to that of O18 at both 10.0 and 2.0ng/ml. A marked decline in activity was observed only below 4.0×10^{-1}ng/ml with baseline activity being approached at 1.6×10^{-2}ng/ml.

d) Two rough LPS from E. coli (J5 and R2) and one from S. minnesota R595 (Re) were assayed for LAL activities. In this instance, O18 LPS produced no plateau at high concentrations (figure 3:41d), but the curve retained a similar shape to that obtained previously. LPS from R595 produced very high activity at 10.0ng/ml, which resulted in a gradual decline to 4.0×10^{-1}ng/ml after which point activity fell more rapidly, although a high activity was seen at 1.6×10^{-2}ng/ml. R2 LPS had low activity followed by a sharp fall to a low at 4.0×10^{-1}ng/ml. J5 LPS had the lowest activity at 10.0ng/ml and this fell gradually to minimal activity at 4.0×10^{-1}ng/ml.

e) As a result of the anomalous result with R595 LPS in (d), LAL activity of this was re-assessed along with that of J5. Results (figure 3:41e) showed that once again J5 possessed very low activity, while R595 possessed greater activity than LPS from O18, with baseline values achieved at 3.2×10^{-3}ng/ml.

f)The results obtained in the above assays with LPS from E. coli O18 were compared in a single graph (figure 3:42) along with a curve of the mean values of LAL activity at each LPS concentration. All curves were sigmoid and values were reasonably similar, although

test-to-test variation was evident.

3:6:2. Inhibitory Action of Human Sera on LAL Activity of Lipopolysaccharides.

An initial attempt was made to inhibit LAL activity of LPS from *E. coli* O18 with the control sera from ELISA assays (GL+ and GL-). A doubling dilution series of LPS from a concentration of 2.0ng/ml downwards was prepared and mixed with each serum (undiluted). Measurement of A_{405} produced the results indicated in figure 3:43. LPS alone showed saturation of activity above a concentration of 1.0ng/ml and below this point activity fell sharply. In the presence of undiluted serum (both GL+ and GL-) LAL activity was completely abolished.

3:6:3. "Endotoxic" Activities of Purified IgG.

The initial attempt at inhibition of LAL activity of LPS from *E. coli* O18 with IgG purified from blood donor sera (figure 3:44a) indicated that the two IgG preparations used possessed massive LAL activity of their own (approximately equivalent to 2.0ng O18 LPS/ml) when used undiluted. A titration curve of IgG was used to determine at which point LAL activity of IgGs became negligible.

A doubling dilution series of five IgG preparations was carried out, and the results (not shown) indicated no reduction of LAL activity at the final dilution (1:64) compared to the initial dilution (1:8). A five-fold dilution series was then carried out with 3 IgG preparations. The results (figure 3:44b) indicated that all three IgGs assayed possessed very high LAL activities, which was reduced to low levels by dilution to 1:625.

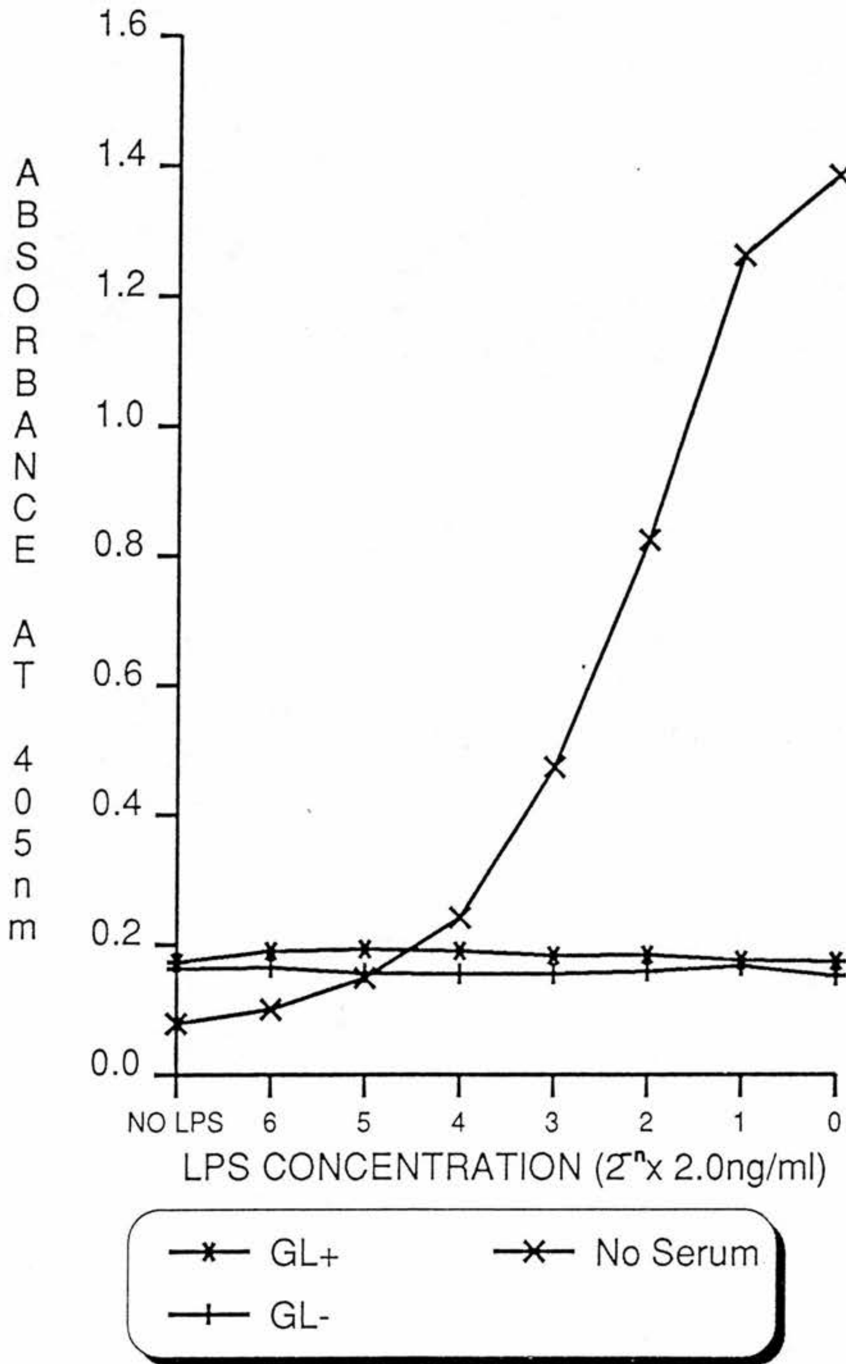


FIGURE 3:43. Inhibitory activities of two human sera with high (GL+) and low anti-CGL (GL-) activities in ELISA upon activation of LAL by LPS from *E. coli* O18.

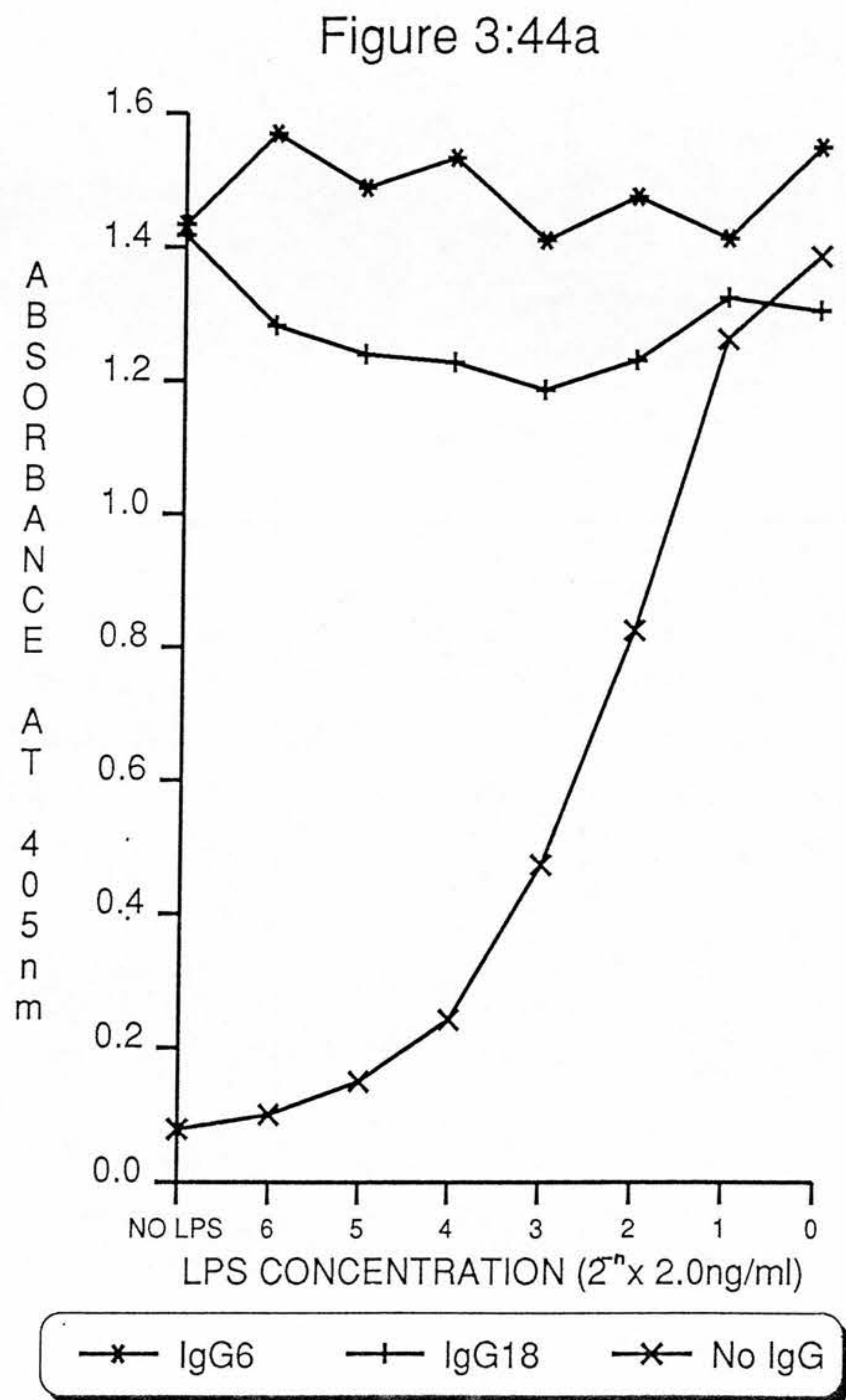


FIGURE 3:44a. Inhibition of the LAL activity of E. coli O18 LPS with two purified IgGs (as described in the key).

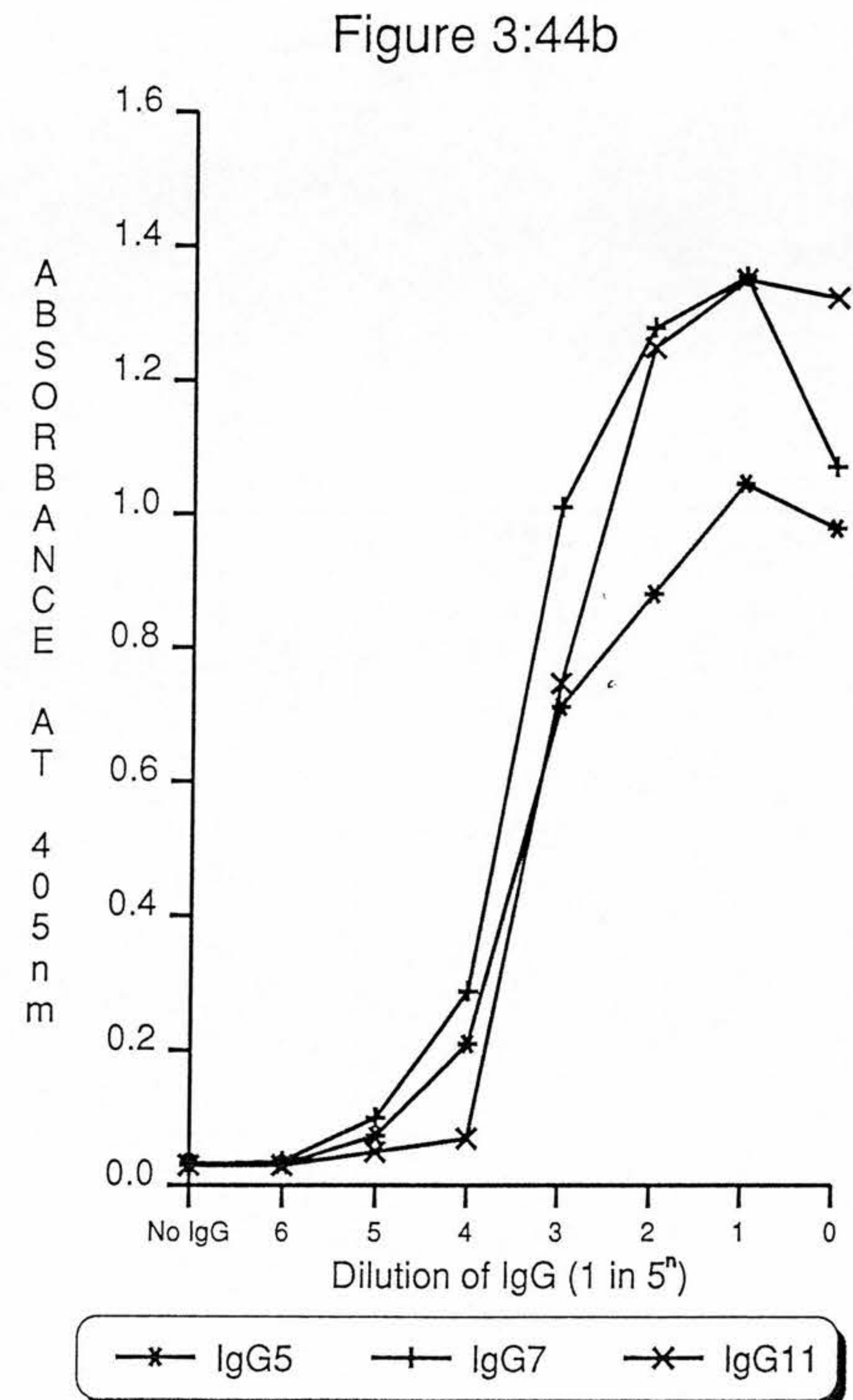


FIGURE 3:44b. Titration of three purified human IgG preparations in a LAL assay. A five-fold dilution series from undiluted IgG (1 in 5^0) to 1:15625 (1 in 5^6) plus a control containing no IgG was used for each IgG.

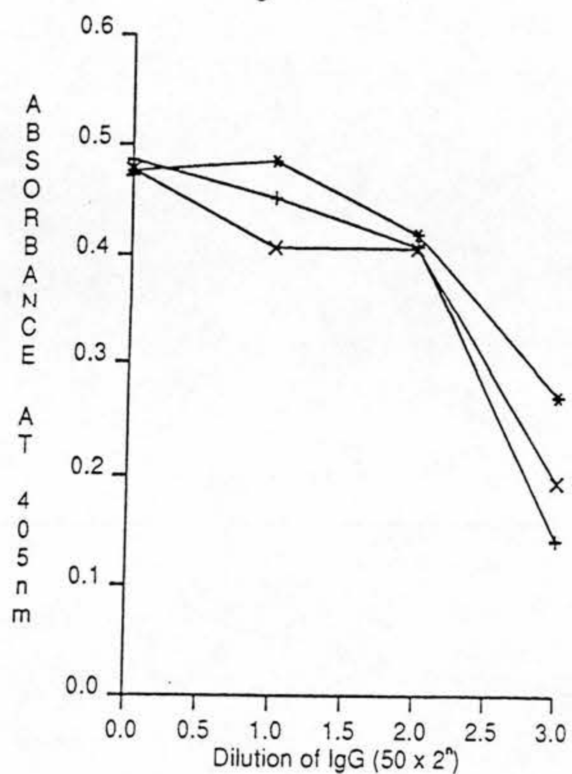
3:6:4. Determination of LAL Activator Present in Purified IgG.

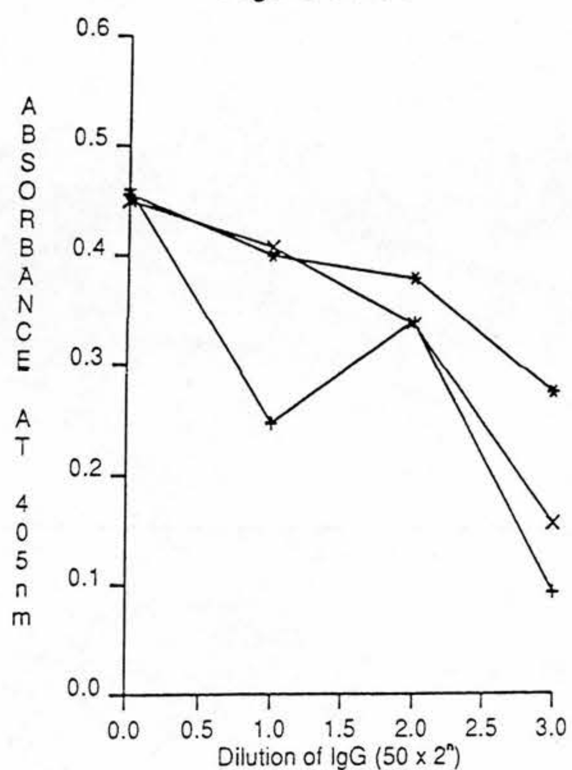
a) Inhibition of LAL activity of IgGs with polymyxin was carried out to determine whether the LAL activity of IgGs was polymyxin-inhibitable (i.e. resided in lipopolysaccharide contaminants). A doubling dilution series of one IgG (no. 7) was prepared from undilute to 1:64 for use as a control curve. Similarly a doubling dilution series of polymyxin was prepared from an initial concentration of 4.0mg/ml. Inhibition was attempted with IgG at a 1:10 dilution versus each concentration of polymyxin. Results indicated once again that activity of IgG in LAL assay was not reduced by a dilution factor of 1:64. Polymyxin at all concentrations appeared to possess no inhibitory activity against IgG.

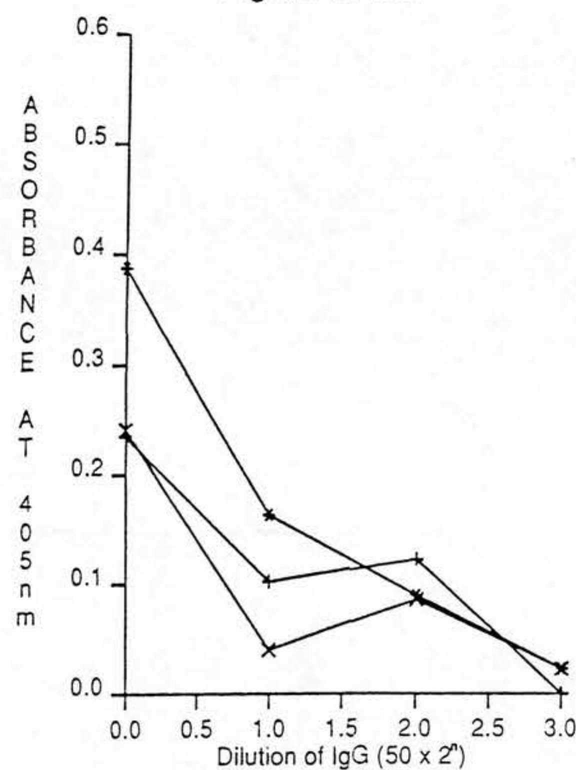
b) The above experiment (a) was repeated with five IgGs. Polymyxin was used at concentrations of 4ng/ml and 40ng/ml, and IgG was diluted 1:50, 1:100, 1:200, and 1:400. An adaptation of the previous LAL assay was used as described in MATERIALS AND METHODS.

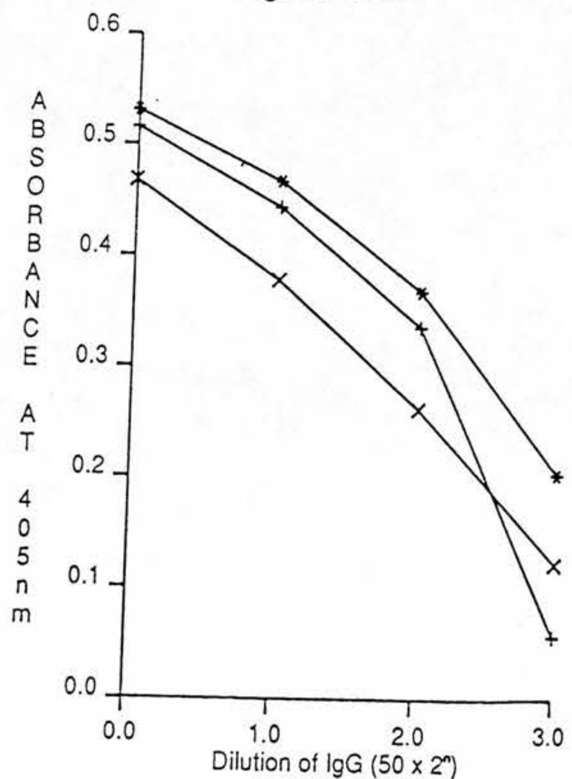
The mean absorbances in this assay of distilled water and polymyxin solutions were all within 0.01 units of each other, therefore polymyxin possessed no LAL activation capacity. Addition of polymyxin to IgGs produced only limited reduction of LAL activation (figures 3:45a-e).

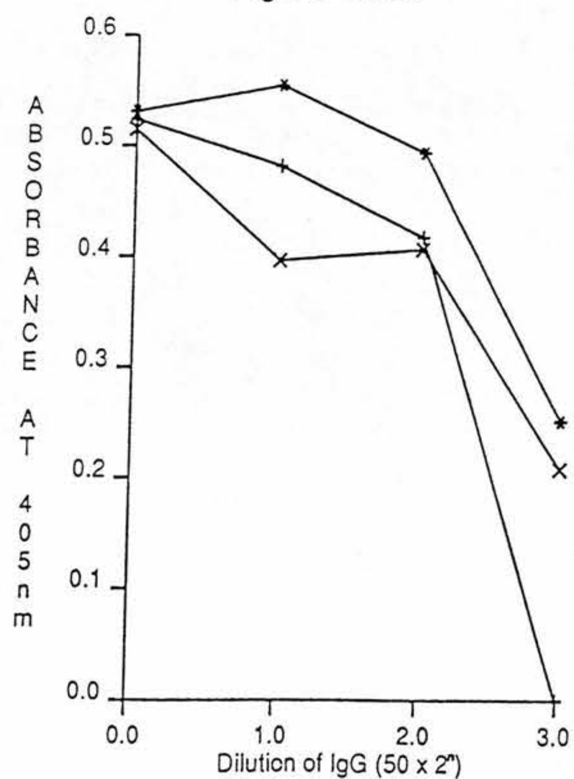
c) IgGs were also subjected to digestion by proteinase K, and samples were separated by electrophoresis through 14% acrylamide separating gel followed by silver staining for visualisation of LPS. No indication of the presence of either R-LPS or S-LPS was obtained by this method.











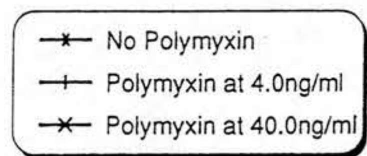


FIGURE 3:45. Inhibition of LAL activity of five purified human IgG preparations with polymyxin. Immunoglobulins were used at dilitions of 1:50, 1:100, and 1:200 against three concentrations of polymyxin. Figures (a), (b), (c), (d), and (e) represent assays carried out with IgG5, IgG11, IgG16, IgG24, and IgG33 respectively.

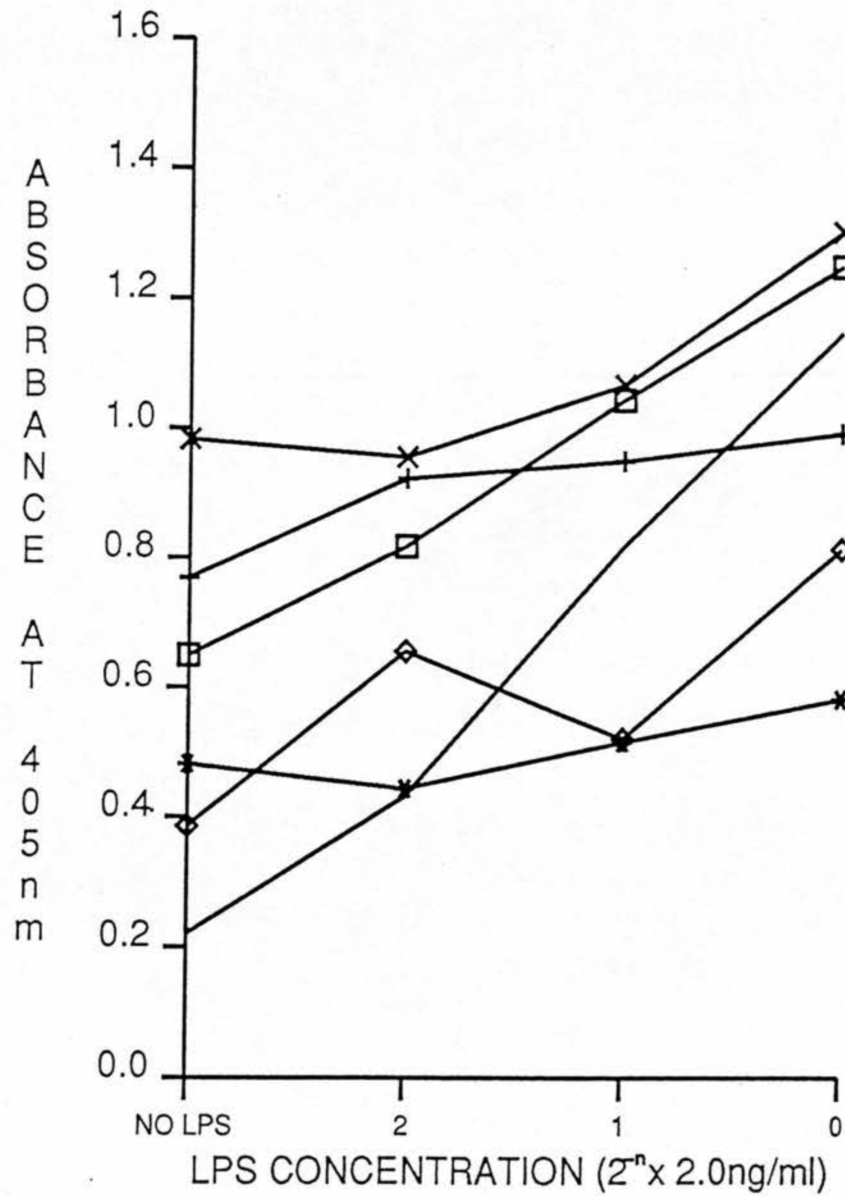
3:6:5. Inhibition of LAL Activities of Purified LPS with IgG.

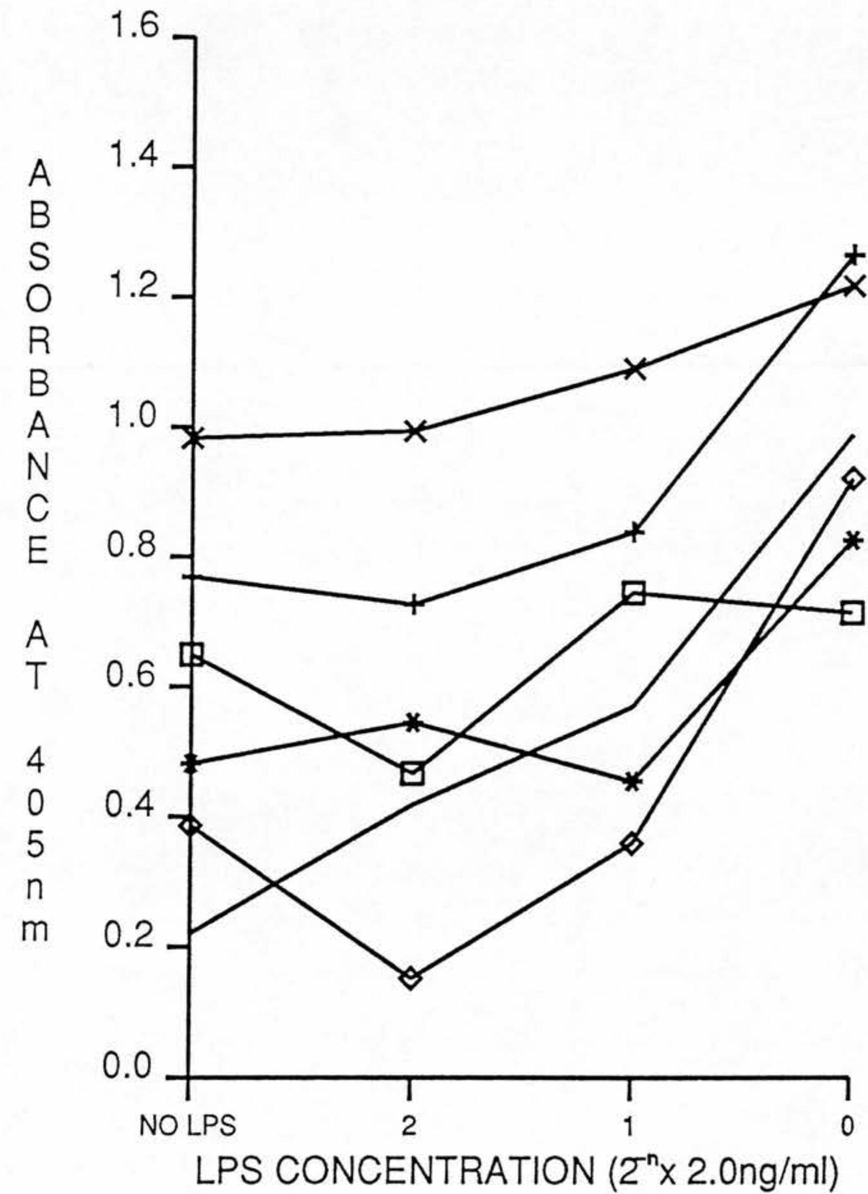
a) Attempts were made to inhibit LPS activation of LAL with IgG purified from human sera. IgG was diluted 1:500 and was mixed *in situ* with LPS in a microtitre plate. Lipopolysaccharides from *E. coli* O18 and *S. typhimurium* R878 and IgG were incubated for 75min at room temperature prior to addition of LAL. The assay was carried out as described in MATERIALS AND METHODS. Even at this dilution some IgG showed reasonably high LAL activity calculated as equivalent amounts of LPS from *E. coli* O18 present in undiluted IgG preparations:

IgG 5 -	approximately	0.10ng/ml
IgG 6 -	"	0.35ng/ml
IgG 7 -	"	1.25ng/ml
IgG11 -	"	0.08ng/ml
IgG24 -	"	0.30ng/ml

Despite this, inhibition was readily demonstrable by IgGs 5 and 11 versus both LPSs (figure 3:46 a & b). IgG24 also showed possible inhibition of R878 LPS when LPS was present at high concentrations (i.e. LPS activity higher than IgG activity in LAL). Similar inhibitory activity in LAL assay was obtained with IgG6 against O18 LPS. IgGs 7 and 24 produced greater LAL activity with O18 LPS at all concentrations than in absence of IgG. A similar increase was observed for IgGs 6 and 7 with R878 LPS.

b) To reduce LAL activity of IgG further, a dilution of 1:1000 was used for IgG5 to inhibit the activities of LPS from *P. aeruginosa* Habs type 1, and *E. coli* O18, O16, and O6. LPS and IgG were incubated for 3h prior to being assayed. LAL activity of IgG was





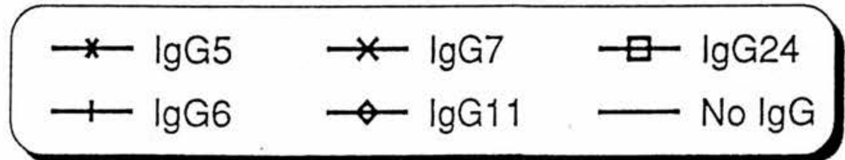


FIGURE 3:46. Inhibition of the LAL activities of lipopolysaccharides from E. coli O18 (figure 3:46a) and S. typhimurium R878 (figure 3:46b) with five purified human IgGs.

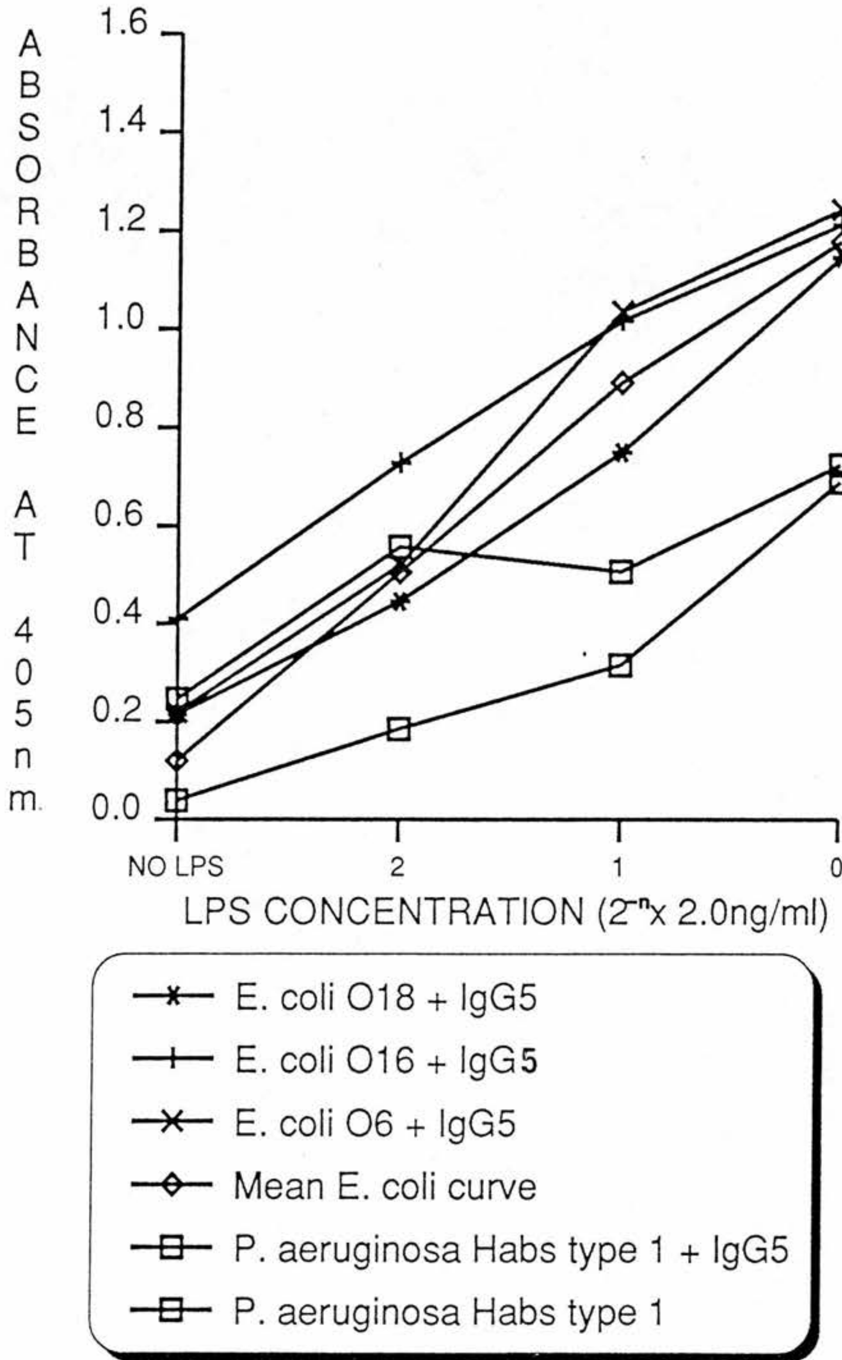


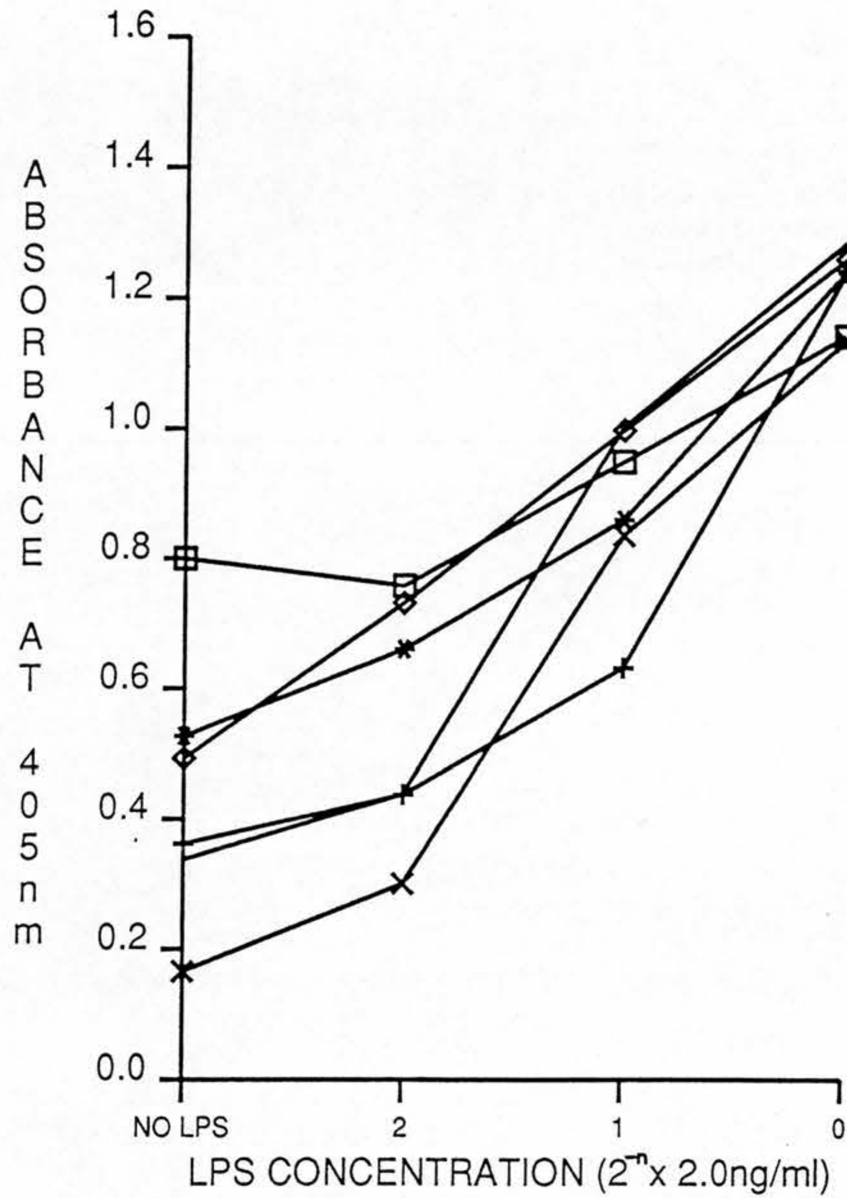
FIGURE 3:47. Inhibitory activity of a purified human IgG (IgG5 at a dilution of 1:1000) on LAL activity of four smooth lipopolysaccharides (as detailed in the legend) in comparison to the activity of P. aeruginosa LPS and the mean activity of E. coli LPS as shown in diagram 3:41b.

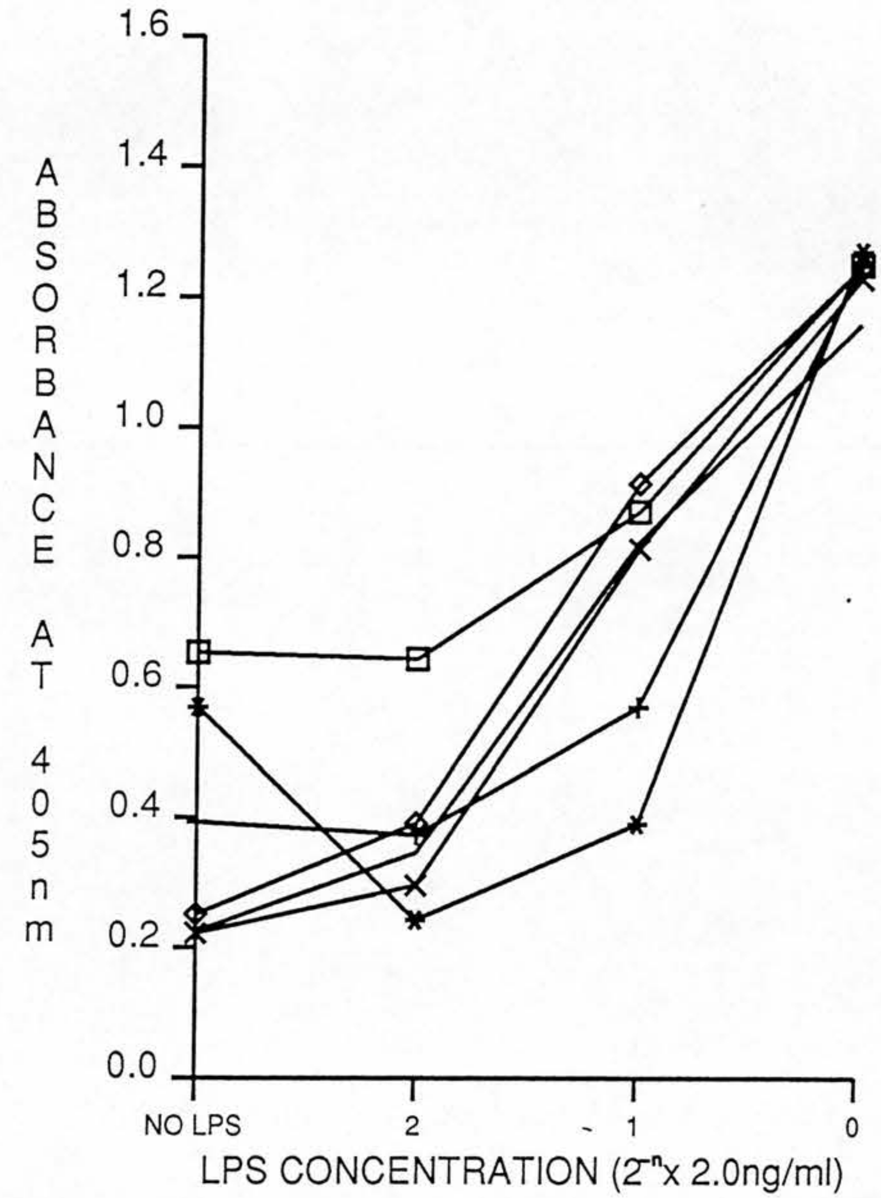
negligible. No significant differences were observed in activities of LPS from the E. coli strains, but minimal inhibition was seen against P. aeruginosa (figure 3:47).

c) E. coli O18 and O16 were used initially with 7 IgGs. Inhibition was carried out with LPS present at final concentrations of 2.0, 0.4 and 0.08ng/ml and IgG at a dilution of 1:500. Pre-incubation of LPS and IgG was carried out for 3h at room temperature before addition of LAL. Inhibition of O18 was produced by IgGs 5, 11, 16, and 33, while IgGs 6 and 7 increased LAL activity of this LPS (figure 3:48a). With O16 LPS, only IgGs 5 and 11 exhibited inhibitory activity (figure 3:48b). The other IgGs had little effect of LAL activity, except IgG6 which caused increased activation of LAL.

d) Inhibition of E. coli O6 and S. typhimurium R1542, R878, and R1102 lipopolysaccharides was attempted with five IgGs. A dilution of 1:500 of IgGs was used throughout versus a variety of LPS concentrations (figure 3:49a-d). O6 was inhibited significantly only by IgG5. R1542 and R878 were inhibited to some extent by all IgGs, but anomalous results occurred at some points (these anomalies were removed upon repetition - figure 3:50). The activity of R1102 was inhibited by all IgGs, with IgG5 being the least effective.

e) A final inhibition was carried out with IgGs at a dilution of 1:500, and LPS at concentrations giving approximately 75% of their maximum activity. The LAL values obtained indicted that many of the predictions of 75% values were inaccurate, but an overall mean of 66.3% of maximum activation was obtained. The inhibitory activities of IgGs in this assay are presented as bar charts (figure 3:51a-g) where 100% represents complete removal of LAL activity by IgG and 0% represents no effect on LAL activity of LPS by IgG. All IgGs





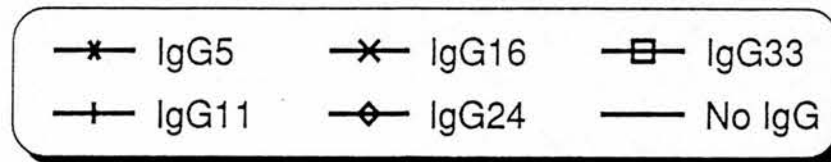
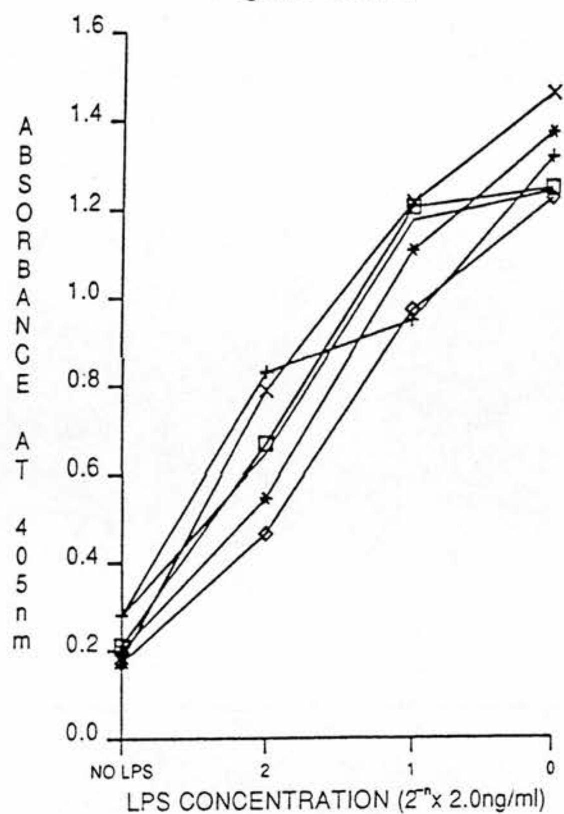
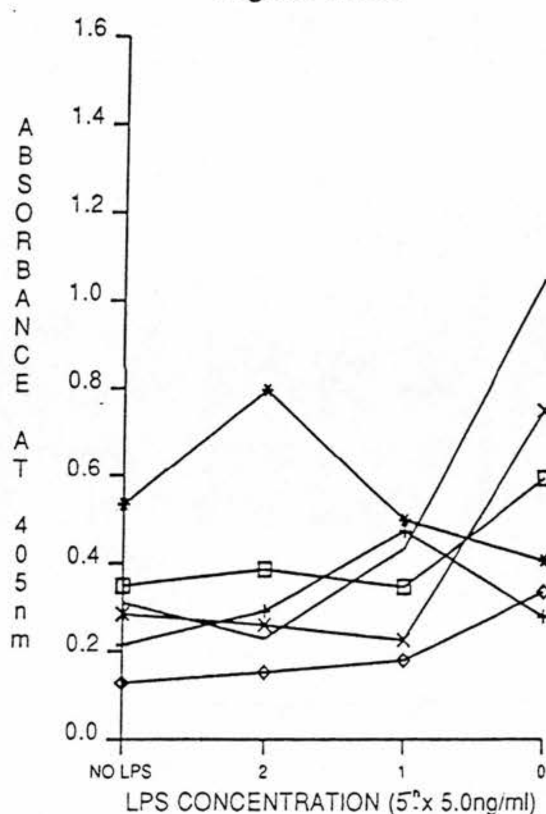
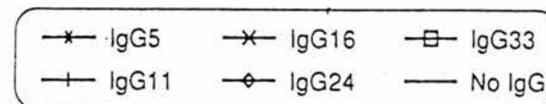
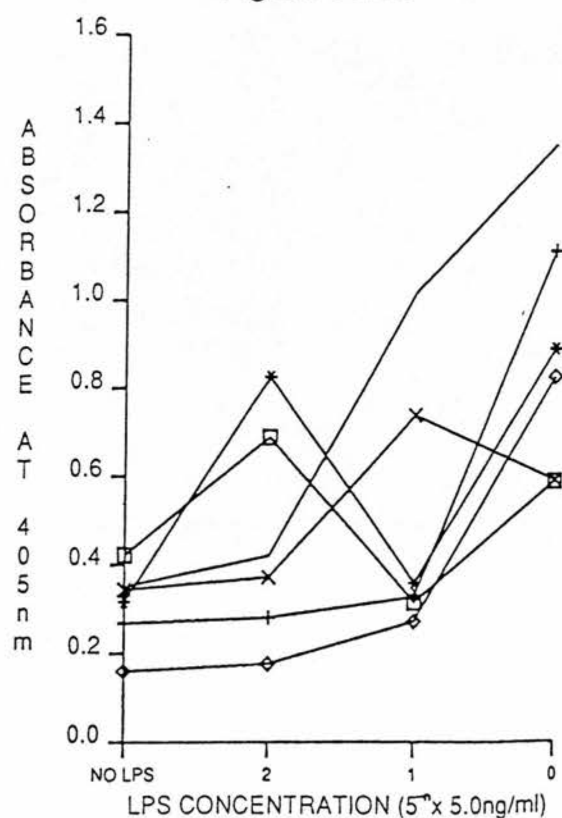


FIGURE 3:48. Inhibition of the LAL activities of lipopolysaccharides from E. coli O18 (figure 3:48a) and O16 (figure 3:48b) with five purified human IgGs.









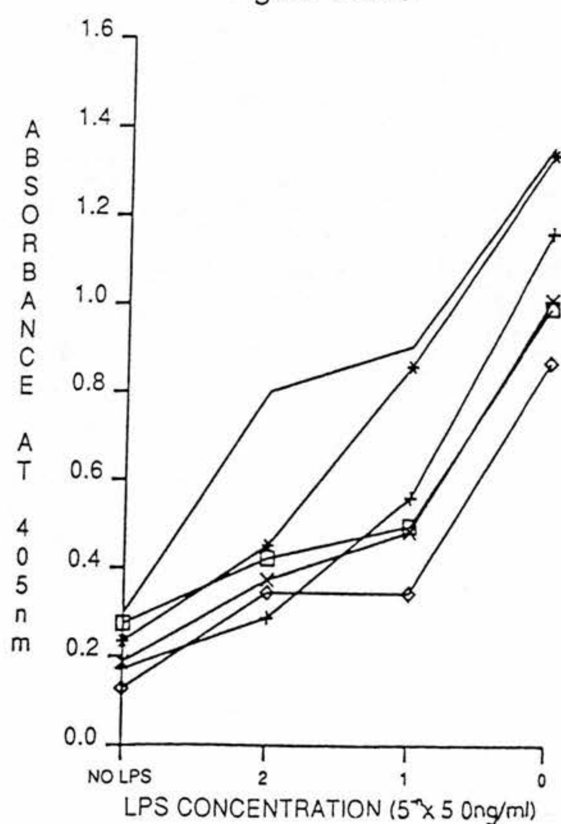
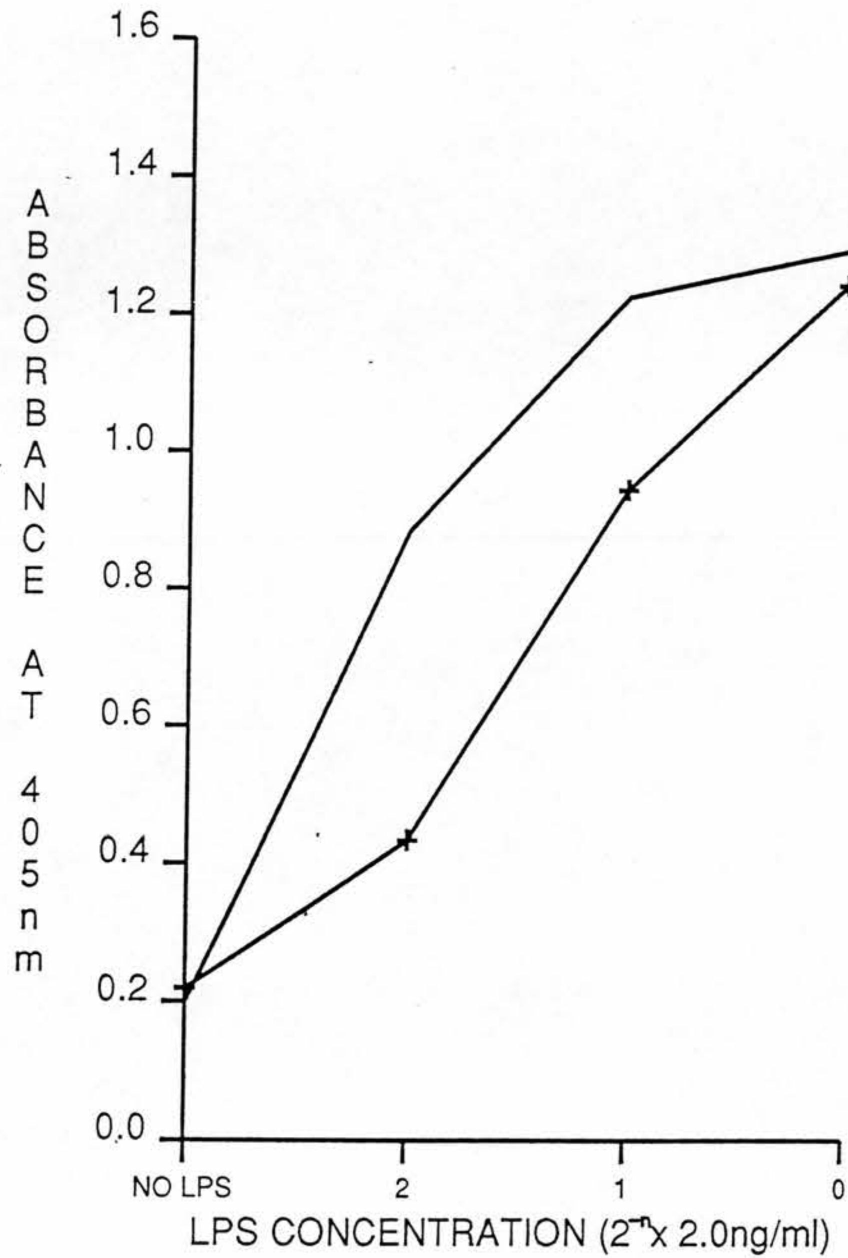
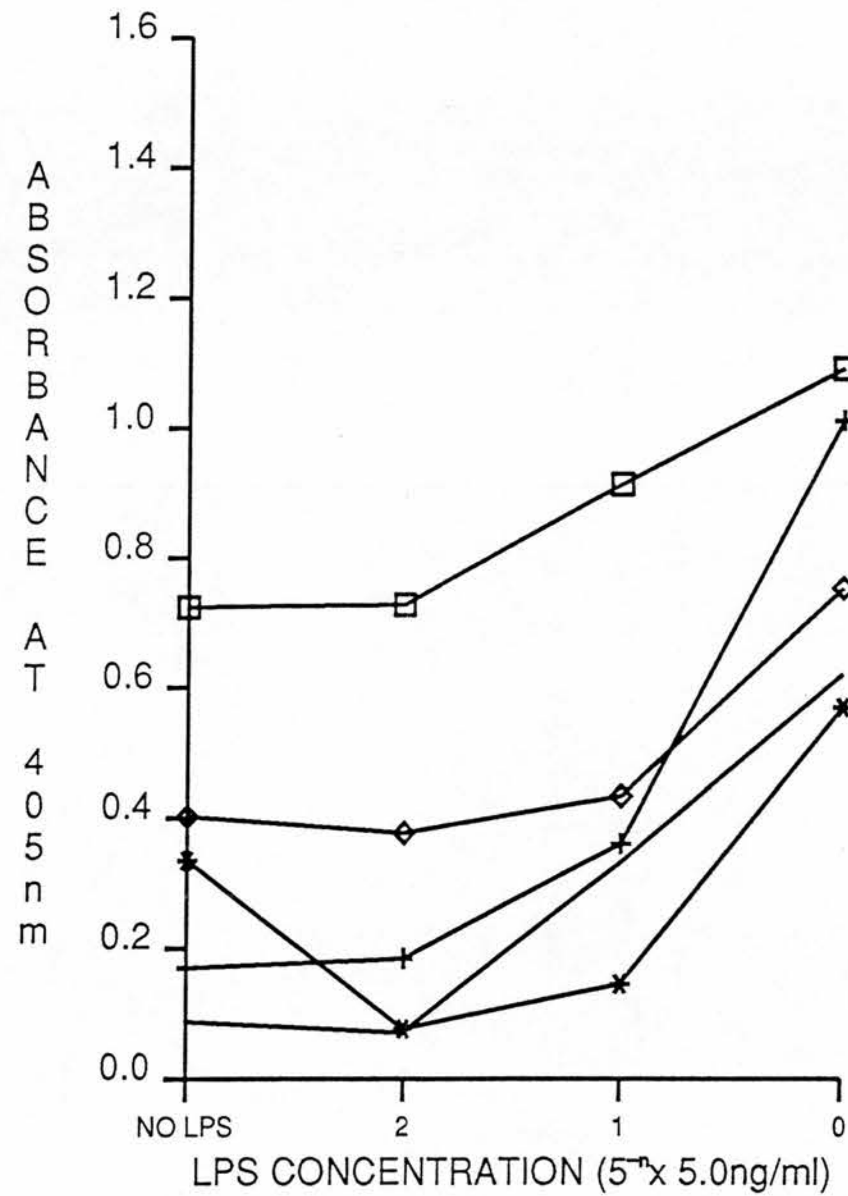
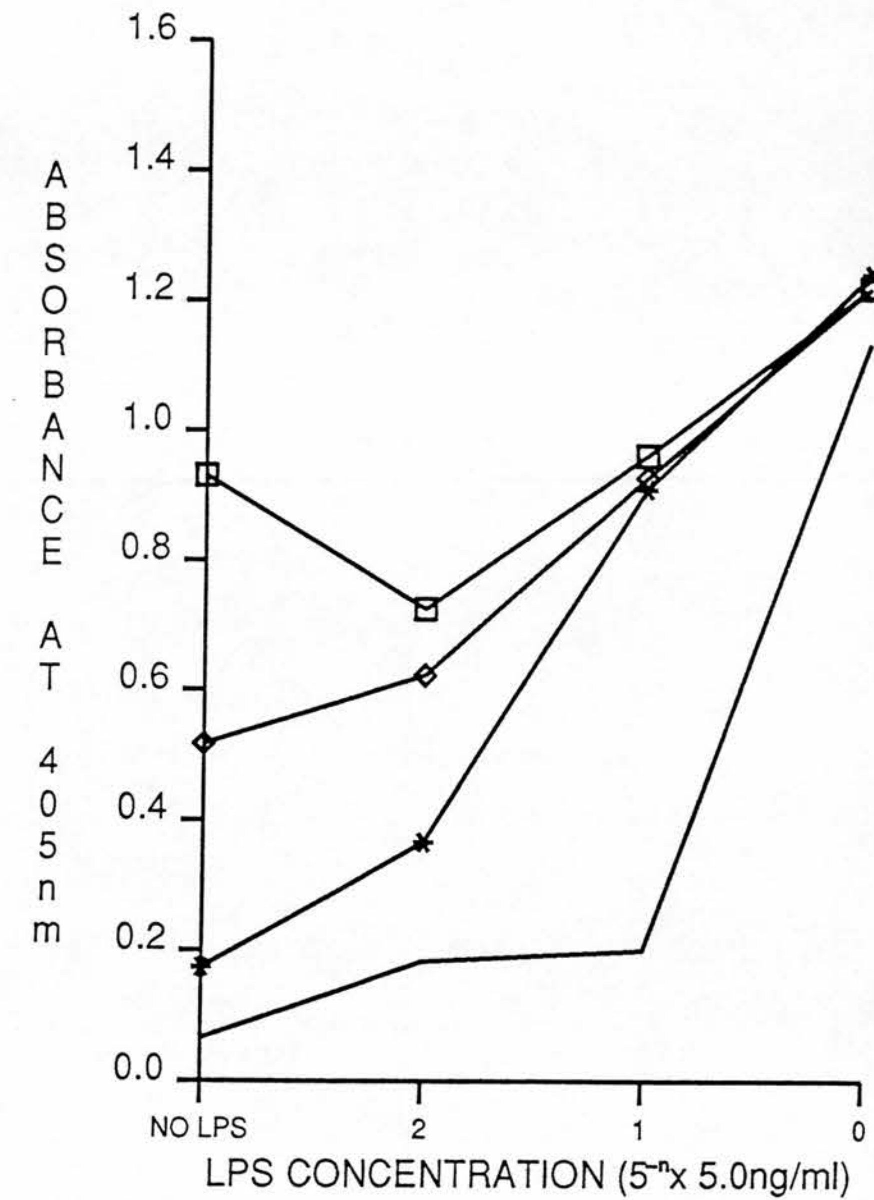


FIGURE 3:49. Inhibition of the LAL activities of lipopolysaccharides from <u>E. coli</u> 06 (figure 3:49a), <u>S. typhimurium</u> R1542 (figure 3:49b), R878 (figure 3:49c) and R1102 (figure 3:49d) with five purified IgG.







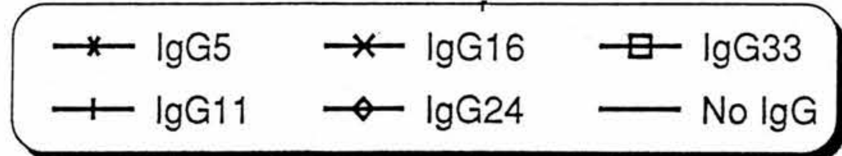
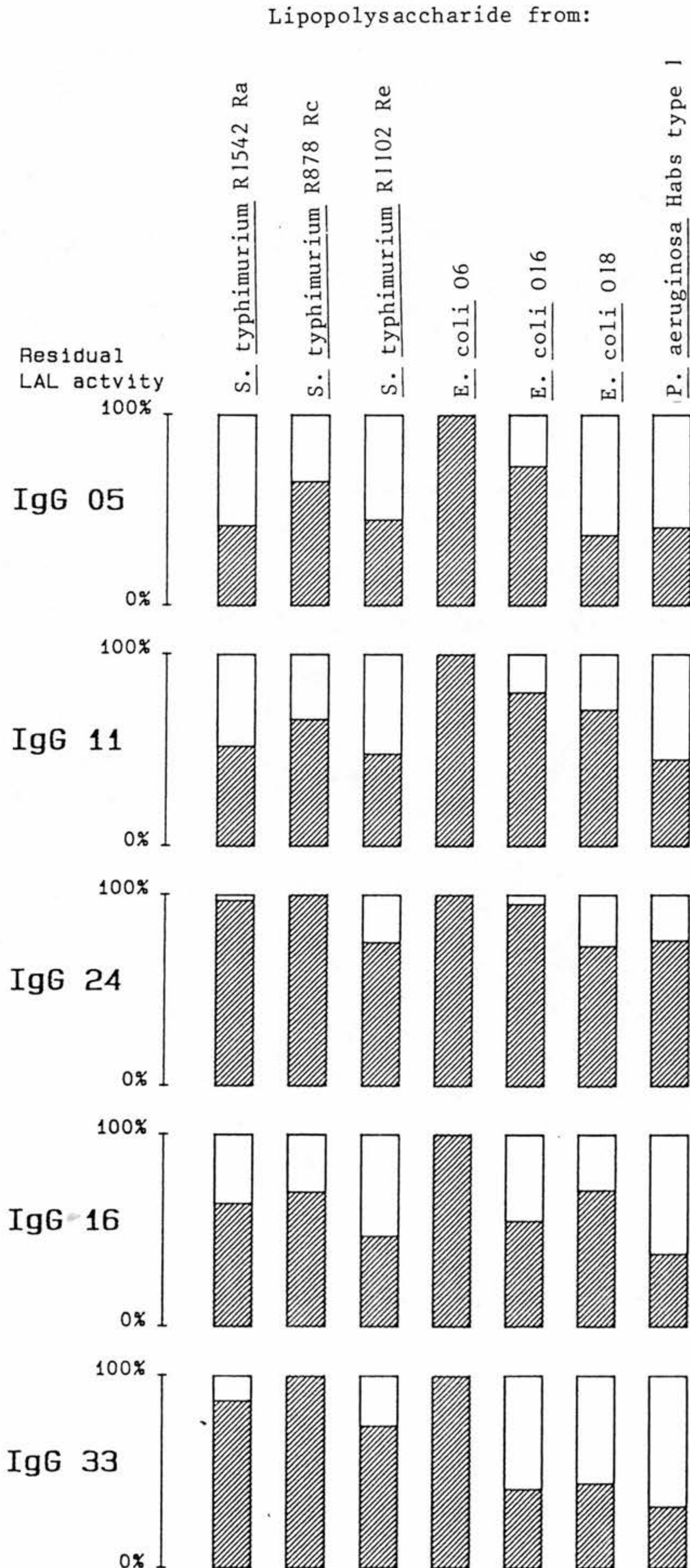


FIGURE 3:50. Repetition of inhibitions shown in Figure 3:49 carried out under identical conditions.

FIGURE 3:51. Inhibition of Limulus Amoebocyte Lysate Activity of Purified Bacterial Lipopolysaccharides with Human IgG Preparations.



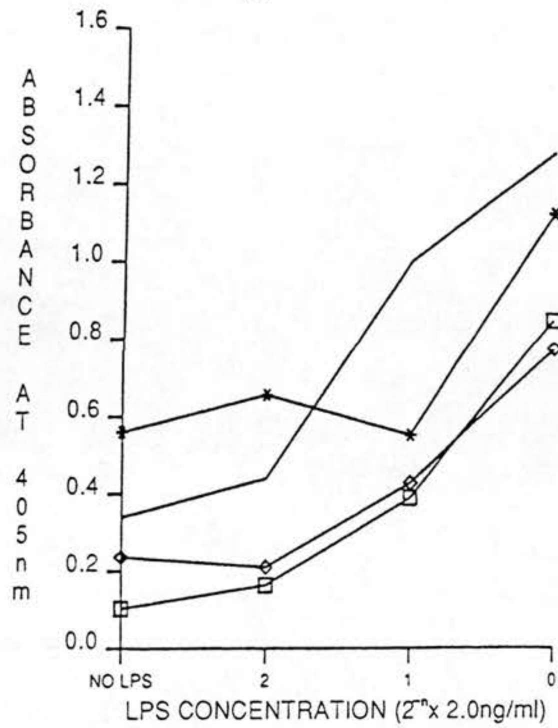
possessed inhibitory activity against most LPS, with much variation present between IgGs.

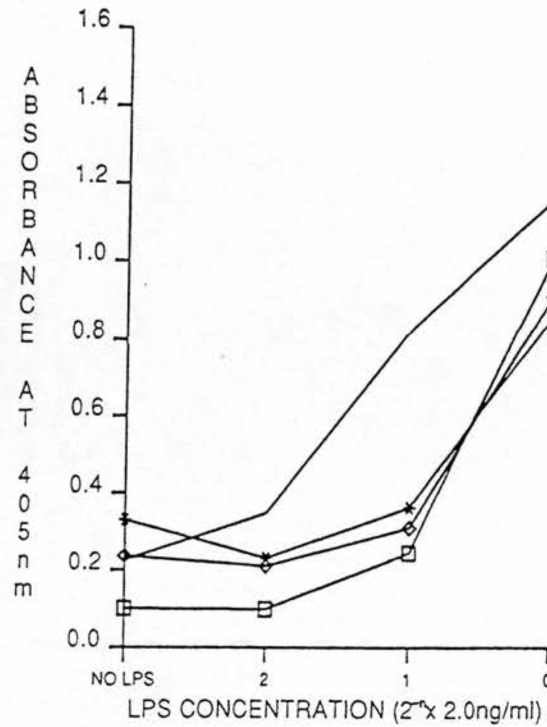
3:6:6. Inhibition of LPS Activation of LAL with Polymyxin.

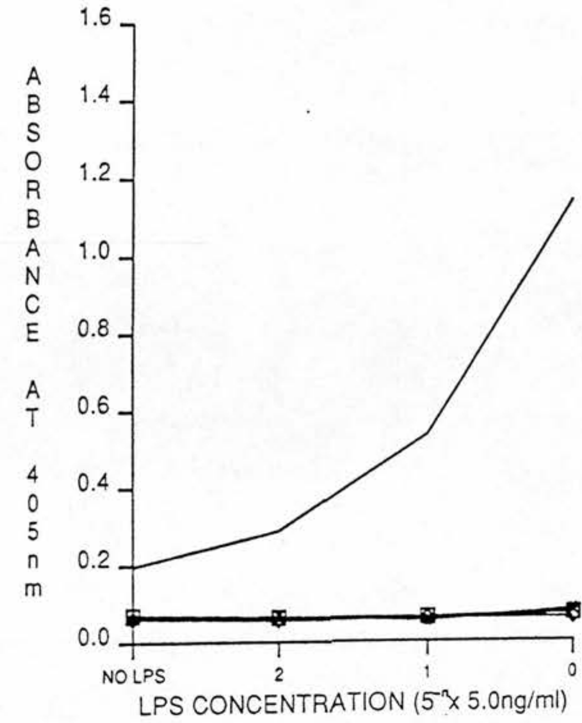
The inhibitory capacity of polymyxin on LPS in the LAL assay was determined by addition of various concentrations of polymyxin to LPS in microtitre plate wells as described above for IgG.

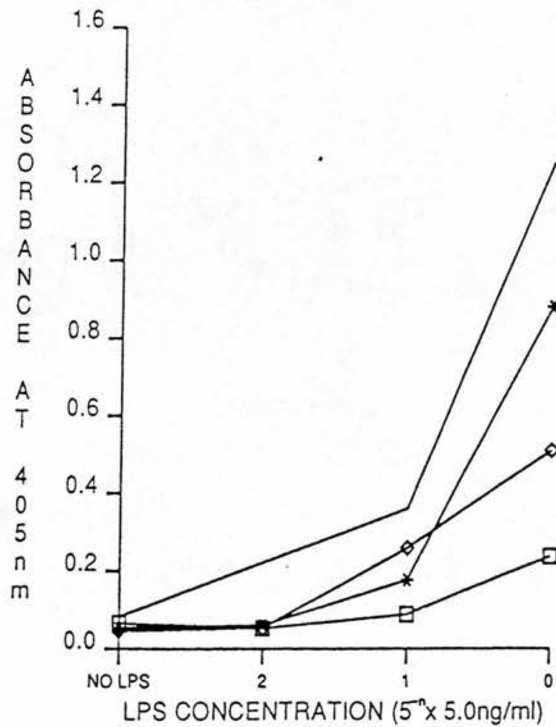
a) The activity of *E. coli* O18 and O16 LPS were assessed in the presence of three concentrations of polymyxin. Polymyxin and LPS were incubated for 180min at room temperature prior to determination of LAL activity. The results (figure 3:52 a & b) clearly show that polymyxin inhibited LPS activation of LAL. Little difference was observed between polymyxin at 4.0 and 40.0ng/ml, but a concentration of 400ng/ml was more noticeable as an inhibitor, especially at higher LPS concentrations.

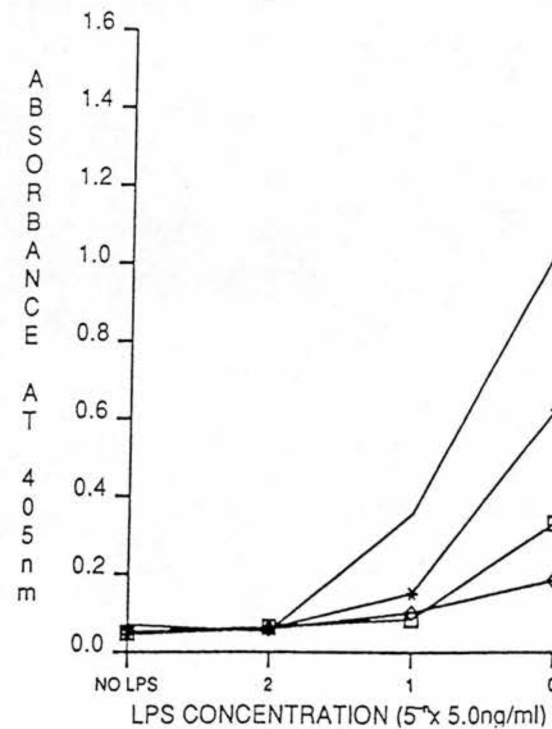
b) Inhibition as described in (a) but with 10-fold higher polymyxin concentrations was carried out on R-LPS from *S. typhimurium* (R1542, R878, and R1102). Figure 3:52 c, d, and e demonstrates that inhibition occurred with all LPS. All concentrations of polymyxin removed totally the activity of R1542 LPS. For both R878 and R1102, increasing concentrations of inhibitor resulted in increased expression of inhibition, and total removal of LAL activity of LPS was not obtained.











Key to figures a & b.

- *— Polymyxin at 4.0ug/ml
- ◇— Polymyxin at 40.0ug/m
- □— Polymyxin at 400.0ug/ml
- —— No Polymyxin

Key to figures c, d & e.

- *— Polymyxin at 40.0ug/ml
- ◇— Polymyxin at 400ug/ml
- □— Polymyxin at 4000ug/ml
- —— No polymyxin

FIGURE 3:52. Inhibition of LAL activities of lipopolysaccharides from *E. coli* O18 (figure 3:52a) and O16 (figure 3:52b), and *S. typhimurium* R1542 (figure 3:52c), R878 (figure 3:52d) and R1102 (figure 3:52e) with polymyxin. Polymyxin was present at 4.0ug/ml, 40.0ug/ml and 400.0ug/ml for figures (a) and (b), and at ten-fold higher concentrations for figures (c), (d) and (e).

3:7. In vivo Activities of Anti-LPS Immunoglobilins.

3:7:1. Determination of Lethal Doses of Bacteria in Swiss White Mice and the Protective Activities of Immunoglobulins.

i) Swiss white mice were initially used for determination of lethal doses of various bacteria. Bacteria were cultured overnight in NB then harvested and washed twice in sterile PBS. The optical density at 600nm (A_{600}) was determined and bacterial counts were calculated by comparison with a standard curve of A_{600} versus colony forming units. A dilution series in PBS was prepared for challenging animals. Swiss white mice were challenged intravenously with 0.1ml of bacterial suspension.

A comparison of the lethal doses of P. aeruginosa Habs type 1 and rough mutant PAC605 was made. The results obtained are presented in the table below.

TABLE 3:8. Comparison of Lethal Doses of P. aeruginosa Habs type 1 and P. aeruginosa PAC605 in Swiss White Mice (groups of 3).

a) P. aeruginosa Habs type 1

Challenge Dose	Cumulative Deaths Day1	Day2	Day3
$1.9x10^{8}$cfu	1	3	-
$9.5x10^{7}$cfu	0	3	-
$4.8x10^{7}$cfu	0	0	0
$2.4x10^{6}$cfu	0	0	0

b) P. aeruginosa PAC605

Challenge Dose	Cumulative Deaths Day1	Day2	Day3
$1.1x10^{10}$cfu	0	2	2
$5.50x10^{9}$cfu	0	0	1
$2.25x10^{9}$cfu	0	0	0
$1.13x10^{9}$cfu	0	0	0

These results clearly showed the difference in virulence between smooth and rough forms of bacteria. The rough strain of P. aeruginosa (PAC605) required greater than 10^{10} organisms to produce even 67% lethality, while Habs type 1 (with O-antigen) required approximately $9.5x10^{7}$ organisms for 100% lethality.

ii) The protective activities of three IgG preparations (Pseudomonas

TABLE 3:9. Protective Activity of Immunoglobulins in Mice against Lethal Challenge with P. aeruginosa Habs type 1.

a) Pseudomonas vaccinees IgG (PsV).

IgG Dilution	Cumulative Deaths* after challenge with $3.4x10^9$cfu		
	Day1	Day2	Day3
10^0	0	1	5
10^1	1	4	4
10^2	1	4	4
10^3	2	5	-
no IgG	3	-	-

b) Pseudomonas positive IgG (Ps+).

IgG Dilution	Cumulative Deaths* after challenge with $1.5x10^9$cfu		
	Day1	Day2	Day3
10^0	0	2	2
10^1	3	5	-
10^2	4	5	-
10^3	1	3	3
no IgG	2	3	-

c) Pseudomonas negative IgG (Ps-).

IgG Dilution	Cumulative Deaths* after challenge with $3.4x10^9$cfu		
	Day1	Day2	Day3
10^0	3	3	3
10^1	3	5	-
10^2	5	-	-
10^3	5	-	-
no IgG	1	3	-

*: five mice in each immunoglobulin group three per control group.

vaccinees IgG - PsV, Pseudomonas positive IgG - Ps+, and Pseudomonas negative IgG - Ps-, see MATERIALS AND METHODS) were assessed versus Habs type 1 challenge. Animals were challenged with 15 to 70 times the dose required to kill 100% of animals. Immediately following bacterial challenge, immunoglobulin (0.1ml) at a range of dilutions were inoculated intravenously. Table 3:9 indicated that limited protective capacity was observed with all IgG preparations. Most activity was obtained with PsV, Ps+ was slightly less effective, and Ps- was least protective.

3:7:2. Assessment of Lethal Bacterial Doses and Protective Activities of Immunoglobulins in C57bl/6 Mice.

The lethal doses of bacteria were also assessed in C57bl/6 mice, which were determined to be more sensitive to Gram-negative bacterial challenge. Bacterial suspensions were prepared as described previously, and mice were challenged intraperitoneally (1.0ml).

P. aeruginosa Habs type 1 was used as challenge organism, after growth into log phase (6h) in two media (NB and MALKA). Table 3:10 presents the data obtained.

TABLE 3:10. Comparison of Lethality of P. aeruginosa Habs type 1 Grown in NB and in MALKA (groups of 4 mice).

a) Nutrient Broth Grown

Challenge Dose	Cumulative Deaths Day1	Day2	Day3
3.70×10^8	4	-	-
9.25×10^7	4	-	-
2.31×10^7	0	0	0
5.78×10^6	0	0	0

b) MALKA Grown

Challenge Dose	Cumulative Deaths Day1	Day2	Day3
1.40×10^8	4	-	-
3.50×10^7	4	-	-
8.75×10^6	0	1	1
2.19×10^6	0	0	0

Calculation of LD_{50} values by the method of Reed and Meunch (1938)

indicated that growth of organisms in minimal medium (MALKA - LD_{50} of 1.39×10^7 organisms) permitted lethal challenge at a dose lower than organisms grown in NB (LD_{50} of 4.63×10^7 organisms).

iv) A series of determinations of lethal doses of a range of bacteria was carried out in C57bl/6 mice. In addition, attempts were made to protect mice with a human donor serum selected for very high levels (602% of GL+ value) of IgG to core glycolipid in the ELISA screen of blood donors (HI-NS). Groups of five mice were challenged i.p. with 0.5ml of graded doses of bacteria in suspension. Bacterial challenge was followed within 5min with 0.25ml of PBS or serum at a dilution of 1:10.

Tables 3:11a-i indicate the results obtained with nine organisms.

TABLE 3:11. Protective Activity of a High Titre Donor Serum (HI-NS) Against Intraperitoneal Challenge with Gram-negative Bacteria.

a) *P. aeruginosa* Habs type 1.

Challenge	Cumulative Number of Deaths					
	PBS-treated			HI-NS-treated		
Dose	Day1	Day2	Day3	Day1	Day2	Day3
2.09×10^{10}	5	-	-	4	5	-
4.18×10^9	4	5	-	1	1	1
8.36×10^8	0	0	0	0	0	0
1.67×10^8	0	0	0	0	0	0
3.34×10^7	0	0	0	0	0	0

b) *E. coli* O18:K⁻.

Challenge	Cumulative Number of Deaths					
	PBS-treated			HI-NS-treated		
Dose	Day1	Day2	Day3	Day1	Day2	Day3
approx. 10^9	4	4	4	2	5	-
2×10^8	0	5	-	0	2	3
4×10^7	0	1	2	0	0	0
8×10^6	0	0	0	0	0	1
1.6×10^6	0	0	0	0	0	0

c) <u>E. coli</u> O6:K5.

Challenge Dose	Cumulative Number of Deaths PBS-treated Day1	Day2	Day3	HI-NS-treated Day1	Day2	Day3
$3.80x10^9$	4	4	4	5	-	-
$7.68x10^8$	5	-	-	5	-	-
$1.52x10^8$	1	5	-	2	5	-
$3.04x10^7$	1	5	-	2	3	3
$6.08x10^6$	0	0	0	0	1	1

d) <u>E. coli</u> O16:K1.

Challenge Dose	Cumulative Number of Deaths PBS-treated Day1	Day2	Day3	HI-NS-treated Day1	Day2	Day3
$4.21x10^9$	5	-	-	5	-	-
$8.40x10^8$	5	-	-	5	-	-
$1.68x10^8$	3	5	-	1	5	-
$3.36x10^7$	0	3	4	1	1	1
$6.72x10^6$	1	1	1	0	0	0

e) <u>E. coli</u> O1:K?.

Challenge Dose	Cumulative Number of Deaths PBS-treated Day1	Day2	Day3	HI-NS-treated Day1	Day2	Day3
$8.70x10^9$	4	5	-	4	5	-
$1.74x10^9$	5	-	-	4	5	-
$3.48x10^8$	0	3	4	0	1	3
$6.96x10^7$	0	0	0	0	0	0
$1.39x10^7$	0	0	0	0	0	0

f) <u>E. coli</u> O12:K?.

Challenge Dose	Cumulative Number of Deaths PBS-treated Day1	Day2	Day3	HI-NS-treated Day1	Day2	Day3
$7.70x10^8$	3	5	-	3	5	-
$1.54x10^8$	1	3	3	1	2	2
$3.08x10^7$	0	0	0	0	0	0
$6.16x10^6$	0	0	0	0	0	0
$1.23x10^6$	0	0	0	0	0	0

g) E. coli O15:K?.

Challenge Dose	Cumulative Number of Deaths					
	PBS-treated			HI-NS-treated		
	Day1	Day2	Day3	Day1	Day2	Day3
4.25×10^9	5	-	-	5	-	-
8.50×10^8	4	4	4	5	-	-
1.70×10^8	0	0	0	1	2	3
3.40×10^7	0	0	0	0	2	2
6.80×10^6	0	0	0	0	0	0

h) E. coli K12.

Challenge Dose	Cumulative Number of Deaths					
	PBS-treated			HI-NS-treated		
	Day1	Day2	Day3	Day1	Day2	Day3
1.05×10^{10}	4	4	4	5	-	-
2.10×10^9	0	0	0	0	0	1
4.20×10^8	0	0	0	0	0	0
8.40×10^7	0	0	0	0	1	1
1.68×10^7	0	0	0	0	0	0

i) E. coli C62.

Challenge Dose	Cumulative Number of Deaths					
	PBS-treated			HI-NS-treated		
	Day1	Day2	Day3	Day1	Day2	Day3
1.30×10^{10}	1	3	4	1	2	2
2.60×10^9	0	1	1	0	2	2
5.20×10^8	0	0	0	0	0	0
1.04×10^8	0	0	0	0	0	0
2.08×10^7	0	0	0	0	0	0

These results indicated that in most cases fatalities could be prevented or at least delayed by the application of a normal donor serum with a high titre of IgG to core-glycolipid epitopes. Protective activity was most obvious for doses between approximately 10^7 and 10^8 smooth E. coli, and for doses between 10^9 and 10^{10} smooth P. aeruginosa. E. coli of O-serotypes 18, 6, and 16 showed slightly lower lethal doses than those of O-serotypes 1, 12, and 15. The rough strains of E. coli (K12 and C62) both showed a far higher dose required to result in death of C57bl/6 mice, (greater than 10^{10}

bacteria) and no protection was obtained for these strains with this serum.

3:7:3. Lethal Doses of Bacteria in C57bl/6 Mice with Mucin and Haemoglobin, and Protection with Immunoglobulins.

The lethal dose of bacteria in C57bl/6 mice was assessed with the mucin-haemoglobin model of infection as detailed in MATERIALS AND METHODS. Mice were challenged intraperitonealy with 0.5ml containing bacteria, haemoglobin, and mucin.

TABLE 3:12. Comparison of Lethal Doses of Bacteria in C57bl/6 Mice - groups of 5 - with and without Mucin and Haemoglobin (Mu/Hb).

a) *P. aeruginosa* Habs type 1.

Challenge	Cumulative Number of Deaths - Mu/Hb			+ Mu/Hb		
Dose	Day1	Day2	Day3	Day1	Day2	Day3
5.08×10^{9}	5	-	-	5	-	-
1.02×10^{9}	4	4	4	5	-	-
2.03×10^{8}	0	0	0	5	-	-
4.06×10^{7}	0	0	0	4	4	4
8.13×10^{6}	0	0	0	0	0	0

b) *E. coli* O16:K1.

Challenge	Cumulative Number of Deaths - Mu/Hb			+ Mu/Hb		
Dose	Day1	Day2	Day3	Day1	Day2	Day3
1.20×10^{11}	5	-	-	5	-	-
1.20×10^{10}	5	-	-	5	-	-
1.20×10^{9}	1	3	3	5	-	-
1.20×10^{8}	0	0	0	5	-	-
1.20×10^{7}	0	0	0	3	5	-

c) *E. coli* O18:K^{-}.

Challenge	Cumulative Number of Deaths - Mu/Hb			+ Mu/Hb		
Dose	Day1	Day2	Day3	Day1	Day2	Day3
3.5×10^{10}	0	0	0	5	-	-
3.5×10^{9}	0	0	0	0	1	2
3.5×10^{8}	0	0	0	2	2	2
3.5×10^{7}	0	0	0	0	1	2
3.5×10^{6}	0	0	0	0	0	0

d) E. coli O18:K1.

Challenge Dose	Cumulative Number of Deaths - Mu/Hb			+ Mu/Hb		
	Day1	Day2	Day3	Day1	Day2	Day3
2.54×10^{10}	5	-	-	5	-	-
2.54×10^{9}	2	5	-	5	-	-
2.54×10^{8}	1	5	-	5	-	-
2.54×10^{7}	0	5	-	5	-	-
2.54×10^{6}	0	3	4	4	5	-

The results pointed to a reduction in the dose of bacteria required to produce fatalities when bacteria were administered in conjunction with mucin and haemoglobin. Non-capsulate E. coli O18 possessed a very high lethal dose in both the absence (greater than 3.5×10^{10} bacteria) and presence (approximately 3.5×10^{10} bacteria) of Mu/Hb. E. coli O16 was only slightly more virulent than $O18:K^{-}$ without Mu-Hb, but the lethal dose was markedly reduced when bacteria were co-inoculated with Mu/Hb. A reduction in lethal dose of P. aeruginosa Habs type 1 of 25-fold was obtained with Mu/Hb. The capsulate strain E. coli O18:K1 was the most virulent of these bacteria, requiring 2.54×10^{7} bacteria for 100% lethality without Mu/Hb. In the presence of Mu/Hb, a bacterial count of less than 2.54×10^{6} was required to cause death of all mice.

vi) In view of the low lethal dose of E. coli O18:K1 when administered with Mu/Hb, a more extensive determination was carried out. The results of this are presented in table 3:13 below. These results indicated that approximately 12 organisms were sufficient to produce 100% lethality with Mu/Hb, although at two points above this (2.95×10^{2} and 5.9×10^{1}) only 80% lethality was achieved.

TABLE 3:13. Determination of the Lethal Dose of E. coli O18:K1 in the Presence of Mucin and Haemoglobin.

Challenge Dose	Cumulative Number of Deaths - Mu-Hb Day1	Day2	Day3	+ Mu-Hb Day1	Day2	Day3
5.76×10^8	nd*	nd	nd	5	-	-
1.15×10^8	nd	nd	nd	5	-	-
2.30×10^7	nd	nd	nd	4	5	-
4.61×10^6	nd	nd	nd	3	5	-
9.22×10^5	nd	nd	nd	nd	nd	nd
1.84×10^5	nd	nd	nd	nd	nd	nd
3.69×10^4	5	-	-	nd	nd	nd
7.37×10^3	4	5	-	nd	nd	nd
1.48×10^3	5	-	-	nd	nd	nd
2.95×10^2	1	4	4	nd	nd	nd
5.90×10^1	2	4	4	nd	nd	nd
1.18×10^1	1	5	-	nd	nd	nd
2.36×10^0	1	3	3	nd	nd	nd
4.72×10^{-1}	1	1	1	nd	nd	nd
9.44×10^{-2}	0	0	0	nd	nd	nd
nil	0	0	0	0	0	0

* nd - not determined

vii) The protective capacity of various immunoglobulin preparations was assessed in the Mu-Hb model of infection with E. coli O18:K1. Mice were challenged with five times minimum lethal dose - 5xMLD (59 organisms) and 25xMLD (295 organisms). Protection was attempted with four normal human sera (GL+, GL-, MED1, and MED2 - see MATERIALS AND METHODS).

TABLE 3:14. Protective Activity of Four Human Sera Against Intraperitoneal Challenge with E. coli O18:K1

Serum	Cumulative Number of Deaths with challenge dose of 5xMLD Day1	Day2	25xMLD Day1	Day2
GL+	0	5	5	-
GL-	2	5	3	5
MED1	4	5	2	5
MED2	5	-	4	5
nil	2	5	3	5

The results obtained indicated that little significant protective

capacity was demonstrable by these human sera in this model, although a limited prolonging of life was produced by GL+ against 5xMLD.

viii) Protection was attempted with GL+ at two dilutions as summarised in table 3:15.

TABLE 3:15. Protective Activity of a Normal Blood Donor Serum (GL+) Against Intraperitoneal Challenge with E. coli O18:K1 in the Mucin and Haemoglobin Model.

Serum Dilution	Cumulative Number of Deaths with challenge dose: 5xMLD			25xMLD		
	Day1	Day2	Day3	Day1	Day2	Day3
1:8	4	5	-	4	5	-
1:32	4	5	-	5	-	-
no serum	4	5	-	5	-	-

No significant protective capacity was observed, but one mouse from a total of five showed extension of survival with a dilution of 1:8 of GL+ against a challenge of 25xMLD.

ix) Two purified IgGs and a monoclonal antibody (versus O-specific antigen) were used to protect C57bl/6 mice against 4 challenge doses of bacteria. IgG were diluted 1:5 to give a concentration of approximately 1.0mg/ml protein, of which 100ug was administered i.p. after bacterial challenge.

From the results presented in table 3:16, it could be determined that both purified IgG preparations showed limited protective capacity against challenge with $1.2x10^{1}$ and with $4.8x10^{1}$ bacteria. The monoclonal antibody specific for the O18 O-antigen produced greater protection than the IgGs at all but the lowest challenge dose, where protection was not acheived by any immunoglobulin.

TABLE 3:16. Protective Activity of IgG Purified from Human Donor Sera against Challenge with E. coli O18:K1 in the Mu/Hb Model of Infection (5 mice per group).

Challenge Dose	Cumulative Number of Deaths											
	IgG 5			IgG 24			SZ.184.2.2.5			PBS		
	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3
1.92×10^2	0	5	-	0	5	-	0	4	5	0	5	-
4.80×10^1	0	4	4	0	4	4	0	3	3	0	4	5
1.20×10^1	0	2	2	0	0	0	0	0	0	0	4	4
3.00×10^0	0	1	1	0	1	1	0	1	1	0	1	1

TABLE 3:17. Protective Activity of Purified Human IgG against Challenge with E. coli O16:K1 in a Neutronenic Model of Infection.

Challenge Dose	Cumulative Number of Deaths in groups of 6 C57bl/6 mice:																	
	IgG 5			IgG 11			IgG 16			IgG24			IgG33			PBS		
	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3
1.24×10^8	0	0	6	0	1	6	0	1	5	0	2	4	0	3	4	0	4	6
6.22×10^7	0	2	2	0	2	3	1	2	4	0	2	4	0	2	2	0	1	2
3.11×10^7	1	2	2	0	4	4	0	0	1	0	0	1	0	0	0	0	1	1
1.55×10^7	0	1	1	0	0	2	1	1	1	0	0	1	0	0	0	0	1	1
7.77×10^6	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0

x) The protective activity of purified human IgG was assessed in a neutropenic mouse model of infection. Neutropenia was induced as described in MATERIALS AND METHODS. The challenge organism was E. coli O16:K1, and the results presented in table 3:17 indicate that in comparison to saline-treated controls, a slight decrease in lethality was observed with administration of 100ug of IgG16, IgG24, and IgG33 in mice challenged with 1.24×10^8 cfu. No other group showed any benefit in administration of IgG.

3:7:4. Lethal Toxicity of Bacterial Lipopolysaccharides.

i) Toxicity of LPS from E. coli O86:K61 was assessed in Swiss white mice. Galactosamine was co-inoculated with LPS as described in MATERIALS AND METHODS. Five mice were present per group and each mouse was administered 0.1ml i.v. The results indicated that with no galactosamine, lethality was not obtained even with 1000ug of LPS. When galactosamine was mixed with LPS, two of five mice died when given 1000ug of LPS, and one of five when administered 500ug LPS. No mice died with 500 or 250ug LPS without galactosamine, or at 250ug with galactosamine.

ii) A comparison was made between different solvents and their effect upon toxicity of S. typhimurium R878 LPS in Swiss white mice in the absence of galactosamine. Three days after challenge, four of five mice administered 2.5mg LPS and 2 of 5 given 1.25mg LPS dissolved in distilled water were dead. Of the mice administered LPS dissolved in PBS, only one of five receiving 2.5mg of LPS died. No other mice died.

iii) Administration of galactosamine (8.0mg/mouse i.p.) to C57bl/6 mice receiving LPS (0.75ml i.p.) from E. coli O86:K61 produced a

TABLE 3:18. Protective Activity of a Normal Human Serum (MED2) against Lethal Intraperitoneal Challenge with LPS from E. coli O18.

a) Female mice (2 per group)

	Cumulative Number of Deaths								
	MED2 1:10			MED2 1:100			PBS		
LPS Dose	Day1	Day2	Day3	Day1	Day2	Day3	Day1	Day2	Day3
100 ng	2	–	–	0	0	0	2	2	2
20 ng	1	1	1	1	1	2	1	1	1
nil	0	0	0	nd	nd	nd	0	0	0

b) Male mice (2 per group)

LPS Dose									
100 ng	1	1	1	2	–	–	2	–	–
20 ng	0	0	0	2	–	–	2	–	–
nil	0	0	0	nd	nd	nd	0	0	0

TABLE 3:19. Protective Activity of a High-Titre Normal Human Serum against Challenge of Mice with LPS from E. coli O18.

a)

LPS Dose	Cumulative Number of Deaths					
	HI-NS 1:10			PBS		
	Day1	Day2	Day3	Day1	Day2	Day3
100 ng	2	2	2	4	4	4
50 ng	1	1	1	3	4	4
20 ng	2	2	3	1	1	1
10 ng	0	0	0	1	1	1

b)

LPS Dose	HI-NS 1:5			PBS		
	Day1	Day2	Day3	Day1	Day2	Day3
100 ng	4	–	–	2	2	2
75 ng	2	3	3	3	3	3
50 ng	2	3	3	3	3	3
20 ng	1	1	1	1	4	–
10 ng	1	1	1	1	1	1

c)

LPS Dose	HI-NS 1:5			PBS		
	Day1	Day2	Day3	Day1	Day2	Day3
100 ng	2	2	2	2	2	4
75 ng	4	4	4	2	2	2
50 ng	1	1	1	5	–	–
20 ng	3	3	3	3	3	3
10 ng	4	4	4	1	1	1

10^5-fold increase in sensitivity to LPS. Two milligrams was required to cause death in mice which did not receive galactosamine. A ten-fold lower dose caused no deaths, but in mice administered galactosamine, 2.0ng of LPS was sufficient to kill 100% of mice.

iv) Protection against LPS challenge was carried out with E. coli O18 LPS (mixed with galactosamine - final volume 0.5ml) in C57bl/6 mice. A normal human serum (MED2) was used initially and dilutions (0.25ml) were administered i.p. immediately following LPS challenge. Table 3:18 presents the data obtained. Two mice were present per group.

These data show that some protection was produced through administration of serum diluted 1:10 in male mice. Protection was also obtained with serum diluted 1:100 against 100ng of LPS in female mice.

v) Further protective studies were carried out with a high titre anti-core glycolipid serum (HI-NS). Five male mice were used per group as summarised in the table below (table 3:19a), and was repeated in male (3:19b, four mice per group) and in female (3:19c, 5 per group) mice.

Much variability was obtained between each of these sets of data. While protection occurred in some situations, other conditions produced greater lethality in groups receiving serum than in those receiving PBS. For two of the groups of data, lethality of LPS in mice treated with PBS showed no pattern consistent with the LPS doses given.

DISCUSSION

4:1. Detection of Human Antibodies against Core Glycolipid Epitopes of Lipopolysaccharide.

There have been two recent reports (Fomsgaard *et al* 1987; Gaffin 1983) of the presence of high levels of anti-LPS immunoglobulins in the blood donor population. Both report similar proportions (5% by Fomsgaard and 7% by Gaffin) of individuals in their surveys who possessed 'therapeutically useful' levels of IgG for the treatment of septic shock, defined as the presence of greater than or equal to 40ug of anti-LPS immunoglobulin per millilitre of plasma. In the present survey, in which the 'therapeutically useful' level of immunoglobulin is defined differently (see below) there are 2.85% of donors who possess 'therapeutically useful' levels of anti-LPS Ig. The assay system used here differs in two major respects:

i) the ELISA system used herein does not directly measure the amount of IgG in micrograms per millilitre. Selection of "therapeutically useful" levels of IgG was made on the premise of selection of hyper-immune globulin, that is individuals possessing five or more times the mean levels of IgG in the population (as determined by measurement of absorbance.

ii) this ELISA system was set up to detect only cross-reactive anti-CGL IgG and not immunoglobulin to O-antigen as in the other reports.

Since it is the core glycolipid which contains the toxic component (lipid A) of LPS responsible for the pathophysiological alterations associated with septicaemia, it would appear that determination of antibodies to this region would provide a more effective

anti-endotoxin. It has also been shown in many studies (see section 1:5:3) that anti-CGL produced by immunisation provides protection against Gram-negative septicaemia, therefore detection of predominantly cross-reactive antibodies would also be advantageous from this point of view. Despite their use of 11 smooth LPS, it was determined that the system used by Gaffin did detect cross-reactive immunoglobulins (Gaffin et al 1985a).

The distribution of IgG to LPS in the current population and in that of Fomsgaard are very similar (figure 3:1).

This LPS-polymyxin ELISA was shown to provide a generally good indication of IgG levels to many lipopolysaccharides and LPS-derived antigens (figure 3:2). It can be seen that sera with high absorbances in the CGL-pool assay tend to possess overall higher antibody levels to other LPS antigens than those sera with lower absorbances in the CGL-pool assay.

The relationship between CGL-pool results and general levels of anti-LPS IgG is also reflected in the results presented in figures 3:3 and 3:4 with serum and purified IgG respectively. Additionally, both sets of data show the wide range within and between individuals in levels of antibodies against many lipopolysaccharide molecules.

Immunoblotting of sera selected for "high" and "low" levels of IgG in the CGL-pool assay produced unexpected results. Certain sera which were determined as "high" showed little or no reactivity with any of the antigens transferred to nitrocellulose, and certain sera designated as "low" possessed antibodies which bound to many of the antigens. Similar lack of reactivity with LPS antigens transferred onto nitrocellulose was obtained by Mutharia et al (1985). Determination of binding characteristics of anti-LPS antibodies in

serum by immunoblotting does not, therefore, match that obtained in ELISA, possibly through alteration of the antigenic conformation of LPS during the processes involved in the electrophoretic transfer.

When applied to eighteen of the thirty-three purified preparations, immunoblotting revealed that no binding was detectable even when the more sensitive alkaline phosphatase and avidin-biotin systems (see MATERIALS AND METHODS) were used. Binding of IgG did, however, occur to dot antigens applied to nitrocellulose, therefore showing that the antigens are either altered during the electrophoresis steps, or that separation of LPS by PAGE effectively reduces the concentration of antigen below the point at which the low levels of antibodies present in sera or IgG preparations can be detected.

Immunoblotting did, though, prove sensitive enough for the detection of antibodies to the O-antigen of P. aeruginosa Habs type 1 in IgG prepared from Pseudomonas vaccinees (PsV - see MATERIALS AND METHODS), Pseudomonas positive donors (Ps+ - see MATERIALS AND METHODS), and from one high titre blood donor serum (RAC+ - see MATERIALS AND METHODS) but not in Pseudomonas negative donors (Ps- - see MATERIALS AND METHODS). Immunoglobulins were also detectable to the O-antigen of E. coli 086 in PsV. This O-antigen has been shown to be highly immunogenic and elicits a strong specific response in rabbits (see section 4:3), and would be expected to produce positive results in immunoblotting if antibodies were present.

It must thus be concluded that immunoblotting as described here does not provide a sufficiently sensitive system for the detection of anti-LPS in normal human sera, although it has proven useful for analysis of reactivity of monoclonal with various LPS (de Jongh-Leuvenink et al 1985; de Jongh-Leuvenink et al 1986; Sidberry

et al 1985). The ELISA CGL-pool assay, however, provides a sensitive indication of the presence or absence of IgG to many LPS, and provides a good basis for the selection of blood donors for production of potentially therapeutic serum and IgG products for the treatment of septicaemia.

4:2. Endotoxin and Anti-endotoxin in Patients with Septic Shock.

No extensive survey has been carried out so far for the determination of levels of endotoxin and anti-endotoxin present in patients during septic shock, although several reports which cover either the aspect of endotoxin levels (see beolw) or of antibodies to a limited range of LPS (Brauner et al 1986; Brauner et al1987; Johns et al 1983; Pollack et al 1983a; Pollack et al 1983b; Young 1972) exist. Endotoxin, as measured by Limulus amoebocyte lysate assays, has previously been shown to be present at various levels in shock patients and in "normal" individuals, with shock patients showing generally higher levels than the more healthy individuals (van Deventner et al 1988b; McCartney et al 1987; Shenep et al 1988). The levels of endotoxin present in shock patients can be seen to vary from day-to-day and even over shorter periods(McCartney et al 1987).

This survey confirms the presence of high LAL activity in serum from patients with septic shock, and also reflects the variability in endotoxic activity in consecutive daily samples.

Two of these three patients (BS and MCC) were repeatedly blood culture negative while patient MCM showed the presence of pneumococcal septicaemia and signs of septic shock on admission. These results indicate that the presence of Gram-negative bacteria is not an essential requirement for endotoxaemia and the production of septic shock. The observation of "aseptic" endotoxaemia and Gram-positive induced endotoxaemia is supportive of results obtained by other groups (Miller & Wenzel 1987; McCartney et al 1987).

When endotoxin levels are considered in conjunction with antibody levels, a complex series of interactions was seen to occur, as many

of the anti-LPS antibody levels detected could be seen to alter with changing endotoxin levels. Of major note was an inverse relationship between many of the IgGs and the levels of LAL activity, and the IgGs to LPS from S. minnesota R5 (Rc) and R595 (Re) appeared to be most intimately associated with the presence or absence of endotoxaemia. The involvement of Re and Rc type LPSs reflects the results obtained in one aspect of the survey of blood donors presented in section 4:1. It has been demonstrated by Barclay and Scott (1987) that two distinct sub-populations, representative of the 'smallest common denominator' of LPS molecule recognised, are present. In a survey of 763 blood donor sera it was found that individuals directed cross-reactive antibodies to either an epitope within the Re core glycolipid - as with patients BS and MCC (comprising lipid A and KDO) or to an epitope within the Rc core glycolipid - as with patient MCM (comprising lipid A, KDO, heptose, and a hexose), but not present in the Re CGL.

Taking the results from patient BS (figure 3:8), it could be seen that levels of IgG to S. minnesota Re were moderate to high throughout the first ten days as endotoxin levels remained low. During the peak of endotoxaemia anti-Re levels fell, reflecting consumption of these antibodies by endotoxin, and as levels of anti-Re fell endotoxin could rise. Antibodies would be produced during this phase, and as levels of IgG increased, endotoxin fell. Between days 17 and 31 there appeared to be a constant flux between anti-Re and endotoxin with IgG levels sufficient to keep levels of endotoxic activity below 1.0EU/ml, and sufficient endotoxin to cause consumption of IgG. Only from day 31 onwards did anti-Re show signs of recovery to levels seen at admission to this study, as endotoxin

remained low. This patient thus showed recovery in levels of IgG, and removal of endotoxin, but this was not reflected in the clinical outcome as the patient died from the pathophysiological changes associated with septicaemia.

The two other patients showed similar relationships between anti-Re (MCC) or anti-Rc (MCM) with levels of endotoxin in the blood. The levels of Rc-reactive IgG in patient MCM plummeted as endotoxin appeared and remained low throughout endotoxaemia. Antibodies binding to Re were not initially present and levels remained low. This patient showed signs of recovery despite high endotoxin levels and was discharged to a general ward where death occurred the following week. It is possible that death resulted from continued endotoxaemia, but serum samples were not continued after discharge, although clinical indications are supportive of death through septic shock induced by endotoxin.

The final patient (MCC) showed continually falling levels of endotoxin in the face of rising levels of antibodies. As IgG against Re LPS was last to recover this suggests that this is the most intimately involved in neutralisation and removal of endotoxin as it continued to be consumed by LPS as other IgG recovered. The increased IgG levels and reduced endotoxin levels were reflected in this patient by complete recovery from septicaemia.

The role of IgM in these interactions has not been assessed because the aim of this group is to provide an intravenous IgG for the therapy of septicaemia, therefore it was relevant to assay only IgG. It is undoubted that IgM must play some role in neutralisation of endotoxin, and this is supported through the protective ability of anti-endotoxin IgM monoclonal antibodies.

The reduction in levels of antibodies to CGL (particularly to LPS from *E. coli* J5 and *S. minnesota* R595) and also O-antigen during the acute phase of septicaemia and the recovery in patients who survived are similar to those obtained previously (McCabe *et al* 1972; McCabe *et al* 1973; Nys *et al* 1987; Nys *et al* 1988; Young 1972). Further support is provided by Baumgartner and colleagues (Baumgartner *et al* 1987a) who determined that the protective agent in an anti-endotoxin antiserum resided in anti-R595 immunoglobulins.

To clarify further this issue, it will be necessary to subject control patients (trauma or surgical patients with no indications of septic shock) as well as further patients with septicaemia to the above series of endotoxin and antibody assays. However, from the study of these three patients, it seems that repletion of septic shock patients with preparations containing high levels of immunoglobulins to the inner core region of LPS would aid in the neutralisation and removal of the trigger of septicaemia - endotoxin - and thus reduce the mortality rate associated with septic shock.

4:3. Immunoglobulin G Response to Lipopolysaccharides.

The longitudinal study of development of IgG to LPS in rabbits immunised with heat-killed bacteria produced a complex series of ELISA results (see section 3:3). Most immunisations produced a noticeable increase in IgG to homologous LPS. In addition, immunisations produced heterologous responses, in part arising through the production of cross-reactive antibodies to conserved epitopes and to some extent as a result of the action of LPS (which would be present in the boiled cell preparations) as a polyclonal B-cell mitogen. This study is longer and more extensive (in both the range of immunogens and the range of antigens in the assay) than any carried out to date (Johns *et al* 1977; Johns *et al* 1983; Mackie *et al* 1982; Michael & Mallah 1982; McCabe 1972; McCabe *et al* 1972; McCabe *et al* 1973; McCabe *et al* 1977). The results for each rabbit can be summarised individually:

a) Immunisation of rabbit 130 (figure 3:11) clearly showed the production of homologous IgG at each immunisation step, though that produced by *S. typhimurium* Rd was small in relation to the others. The small rises provoked by hydrolysed bacteria to many antigens (possibly through polyclonal B-cell activation) were boosted with Re immunisation. Each immunisation (particularly Re and Rb) produced increases in Ra reactive IgG, which reached maximal levels by the penultimate immunisation (Rb). This would tend to indicate that Ra and Rb possess a dominant common epitope within the sugar units distal from lipid A.

The responses to R-LPS of identical chemotypes from *S. typhimurium* and *S. minnesota* show marked differences with little response detectable to *S. minnesota* antigens. This indicates that the rough

lipopolysaccharides from these two species are not antigenically identical, and their structures must therefore differ. This contradicts a wide body of accepted literature which states that rough LPS molecules from all Salmonella spp. are identical. The small increases obtained to S. minnesota R-LPSs could, however, have appeared through production of a cross-reactive anti-lipid A response after the initial immunisation which increases following all immunisations. The obvious antigenic differences between these two species are reflected in the results obtained in screening of the blood donor population for anti-CGL where once again levels of IgG to S. typhimurium and S. minnesota were not equivalent. These antigenic differences may reside in the quantity and quality of ethanolaminyl and phosphoryl groups present on the mutants of each species.

b) Rabbit 131 produced results (figure 3:12) indicating that the Ra core from S. typhimurium R1542 and those from E. coli R2 and R4 are antigenically very similar. This is borne out through reference to the accepted structures of these lipopolysaccharides as presented in figure 1:4.

Ra-type bacteria induced a specifically outer core response as well as a slight anti-lipid A response, indicating that the lipid A of both S. minnesota and S. typhimurium may be similar structurally and antigenically.

The increase in IgG to many S-LPS as well as that of S. minnesota Ra tends to follow closely the production of lipid A reactive IgG, with the exception of S. typhimurium wild type which more closely follows its Ra mutant. This pattern seems to indicate that access of anti-lipid A antibodies is not restricted by the presence of

O-antigen in purified lipopolysaccharides, and reiterate the cross-reactivity observed between S. typhimurium R-LPSs and S. minnesota lipid A.

Immunisation with Rb once again increased IgG levels to Ra, thereby providing support for the existence of a cross-reactive immunodominant epitope present on both LPS types. Immunisation with lipid A produced a low level, widely reactive response but other R-LPS produced little homologous or heterologous response although immunisation with Rb and Rc produced a small degree of reactivity to P. aeruginosa PAC605 LPS. This may occur through the terminal glucose present on all three of these molecules (see figures 1:3 and 1:4).

These results indicate that the response to the initial immunogen (Ra) was the only major response which occurred, little being observed to many other R-LPS and S-LPS except two E. coli core types.

c) The third rabbit (number 132) showed noticeable levels of IgG prior to immunisation to several antigens E. coli 02, 075, 086 and 0111; P. aeruginosa PAC605; S. typhimurium wild type; and S. minnesota Ra and Rb), therefore response to these would be expected to be a rapid and immediate anamnestic response. These secondary responses were observed for four of these antigens (E. coli 02, 075, and 086; and S. typhimurium wild type) with above baseline values before immunisation, and appear to have occurred through the induction of cross-reactive IgGs although the homologous response was modest.

E. coli J5 was seen to possess epitopes which boost responses to the R2, R3, and R4 core types of this species, but the structure of J5

(reviewed by Barclay & Scott 1987 - see figure 1:3) is dissimilar to the accepted structures of R2 and R4 and appears to possess an unique immunodominant epitope (Appelmelk *et al* 1986a; Appelmelk *et al* 1986b; Baumgartner & Glauser 1987a; Sakulramrung & Domingue 1985; Schwartzer *et al* 1987). The cross-reactivity observed with J5 LPS may thus result from induction of antibodies which bind to Re-chemotype LPS (Baumgartner & Glauser 1987a). Cross-reactivity of J5 LPS was also observed with several *E. coli* O-antigens (O2, O6, O75, and O86). Relation of the sugar compositions of these LPS molecules (Orskov *et al* 1977) indicate similar sugar compositions and therefore possible cross-reactive sites, but polyclonal activation of B-cells appears to be a strong possibility.

The response to the core oligosaccharide of *K. aerogenes* rough mutant (M10B) indicated that cross-reactivity exists with *S. typhimurium* Rc and Rd, as well as *E. coli* R1, R2, R3, and R4. Because of the reactivity with Rd, this indicates that the cross-reactivity results from the heptose region and thus the inner core region of this LPS appears to be similar to that of other enterobacteria.

The presence of unique epitopes on *P. aeruginosa* PAC605 LPS determined by structural analysis (Rowe & Meadow 1983 - see figure 1:4) was supported antigenically through production of a mainly specific response, though some cross-reactivity was observed with cores from *K. aerogenes*, *S. minnesota* Ra, and *E. coli* R1, R2, and R4 though this is not reflected structurally.

Immunisation with two Re type LPSs (*E. coli* F515 and *S. minnesota* R595) produced much polyclonal response with cross-reactivity to many rough LPS evident, thus epitopes present in these Re molecules

(or similar epitopes) are present on LPS from S. minnesota, S. typhimurium, E. coli, K. aerogenes, and P. aeruginosa.

d) In the fourth rabbit (number 143 - figure 3:14) E. coli O18:K^- produced very little antibody response to any LPS.

Lipopolysaccharide from E. coli O6 was highly immunogenic, producing a strong response to the homologous antigen and that from serotypes O2, O16, and O75 as well as S. typhimurium wild type. This cross-reactivity is not borne out by previously reported cross-reactions (Orskov et al 1977). Since responses to S. typhimurium Ra, S. minnesota lipid A, E. coli R2 and R4, and to a lesser extent R3, it appears likely that many of these reactions are occurring through production of anti-CGL antibodies, though strong reactivity has been observed between O6 and O16 with a monoclonal antibody raised against O16.

All other immunisations produced mainly homologous responses although some cross-reactivity with other O-serotypes and some core types was obtained. E. coli O16 did, however, produce a large anti-core response to S. typhimurium Ra probably through cross-reactivity with E. coli R2, R3, and R4, which were also increased.

e) Once again LPS from E. coli O18 was shown to be of low immunogenicity following even two consecutive immunisations, a similar occurrence to that found by Elkins and co-workers (Elkins et al 1987a; Elkins et al 1987b) where certain O-antigens were found to be only weakly immunogenic in mice, and for which a mechanism was proposed. Some heterologous responses were obtained to S. typhimurium Ra, Rb, and wild type; S. minnesota Ra and lipid A; E. coli R2, R4, O2, O12, O75, O86, and O111; P. aeruginosa PAC605. The

reactivity with core types R2 and R4 would seem to point to O18 possessing one or other of these, or a core glycolipid with an epitope which is common to both. The presence of many cross-reactive IgG would seem to be responsible for many of the reactivities observed.

Lipopolysaccharide from P. aeruginosa Habs type 1 was highly immunogenic, producing a predominantly homologous response. Similar strong immunogenic activity has been observed previously through immunisation of mice with P. aeruginosa (Mackie et al 1982).

The cross-reactivities observed by immunisation with E. coli O6 between O-serotypes O2, O6, and O16, as determined above, were confirmed.

The reduction in IgG following the final immunisation may point to the presence of viable organisms in this preparation, though levels recover.

In conclusion, the results produced by this study are summarised as follows:

i) S. typhimurium Ra and Rb possess a strongly cross-reactive epitope common to both LPS.

ii) LPS from equivalent rough mutants of S. minnesota and S. typhimurium are not antigenically identical.

iii) The rough LPS from S. typhimurium Ra and Re possess a common cross-reactive epitope through lipid A and/or KDO.

iv) The cores of E. coli R2 and R4 are strongly cross-reactive with the Ra core of S. typhimurium and are therefore antigenically similar.

v) Rough LPS from P. aeruginosa PAC605 and K. aerogenes M10B are cross-reactive with certain enterobacterial cores.

vi) The O-antigen from *E. coli* O18 is non-immunogenic in rabbits.

vii) *E. coli* O6 and O16 possess strongly antigenically cross-reactive O-antigenic structures.

viii) *P. aeruginosa* Habs type 1 has a strongly immunogenic and immunodominant O-antigen.

4:4. Antigenic Presentation of Lipopolysaccharide in ELISA.

A series of absorptions followed by ELISA, and inhibition of ELISA reactivity with soluble antigens were carried out to determine whether LPS-polymyxin complexes presented an antigenic configuration which was comparable to those observed in other LPS preparations. This determination is necessary firstly because polymyxin may bind to antigenic sites of LPS (Moore et al 1986) thereby obscuring potentialy reactive sites, and secondly to indicate whether use of these complexes is an appropriate means of identifying antibodies which are reactive with LPS as presented in intact organisms. This would therefore indicate the relevance of this assay for determination of anti-CGL antibodies in the blood donor population.

a) The results from the titration of the four sera against four LPS-containing antigen preparations indicated that uncomplexed LPS and OM were most reactive in ELISA. It might have been expected that OM would produce greater reactivity with sera because of the presence of outer membrane proteins to which antibodies could bind. This may indicate a less efficient binding of OM to ELISA strips because of its higher solubility than LPS but as protein-containing antigens are known to bind very efficiently to polystyrene ELISA strips, the lower reactivity with OM may be the result of underestimation of LPS content in OM by the carbohydrate assay. A further surprising result was the relatively low absorbances produced with heat-killed cells as antigen as these again contain many antigens other than LPS. One possibility would be that LPS content was under-estimated by the carbohydrate assay, or that heating of the bacteria at $100^{o}C$ for 10min alters the antigenic structures on the surface thereby preventing interaction with

immunoglobulins. It is also suggested that binding of whole bacteria to ELISA strips is more efficiently facilitated by centrifugation of plates (B. Scott, personal communication), thus providing a further possibility for improvement. Finally, LPS-polymyxin complexes were the least reactive with sera, possibly resulting from their high solubility, and thus lower deposition onto well surfaces. Despite the lower absorbance values observed, LPS-polymyxin was shown to present a more stable antigen in ELISA than purified LPS (Scott & Barclay 1987), and thus the high values obtained against LPS may not have re-occurred upon repetition of coating.

b) The absorption of sera with heat-killed S. typhimurium R878 bacterial cells (section 3:3) showed that all forms of antigen reflected the reduction in IgG levels after each absorption. Similar patterns of reduction in IgG were observed for each antigen preparation.

The marked reduction in IgG reactive with uncomplexed LPS showed that LPS-specific immunoglobulins were present in each serum at reasonable concentrations, and it must be assumed that removal of anti-LPS during absorption accounts for some reduction of IgG seen against OM and heat-killed bacteria.

One serum (GL-) produced low reactivity against all antigens, and reduction in IgG was low after absorption, therefore this serum appears to possess generally low levels of IgG to all S. typhimurium R878 antigens and not only the LPS.

Reduction in IgG was less noticeable with LPS-polymyxin than with uncomplexed LPS, but this may be accounted for by lower concentration of complex being deposited in the wells during the coating process. This would imply that LPS-polymyxin is in a form

which may differ from all other forms of LPS in this assay. However, the relative absorbances produced against each antigen with each serum at all dilutions produced similar relative values in comparison to that of LPS-polymyxin. It can thus be said that the complex presents LPS in a manner which is appropriate for the measurement of anti-LPS antibodies.

c) Absorption of heat-inactivated sera with viable organisms (figures 3:21 to 3:24) also showed reductions in IgG levels following absorption, but large fluctuations were obtained between consecutive dilutions and between successive absorptions, thus reduction in levels of IgG were less well defined than above.

At a serum dilution of 1:50, high absorbances were observed on strips which received only post-coat (and thus contained no antigen). Heat-inactivation of serum must therefore alter the binding characteristics of immunoglobulins thereby permitting non-specific binding to ELISA strips. It is, however, possible that the heat-inactivation process may have altered the IgM present in the sera, thus allowing its binding to microplates either directly of via antigens and it is possible that the heating may also have permitted recognision of IgM by the secondary antibody (anti-human IgG). Further possible contributors to this non-specific binding may be proteins or lipoproteins which bind LPS and which may precipitate onto microplate wells once complexed with LPS.

Reduction of non-specific binding to background levels occurred by a dilution of 1:100, indicating that the components responsible for non-specific binding are present at fairly low levels.

As a result of the problems encountered with heat-inactivated serum in these absorptions, it is recommended that untreated sera are used

for all absorption studies.

d) Inhibition of ELISA was carried out to determine whether antigen present in the aqueous phase could prevent the binding of immunoglobulins to solid-phase rough LPS antigens. Attempts were made to inhibit binding of antibodies to four solid phase antigens (LPS-polymyxin, uncomplexed LPS, OM, and heat-killed bacteria) with three inhibitors (LPS-polymyxin, uncomplexed LPS, and OM).

The initial time-course experiment indicated that co-incubation of inhibitor (LPS) with serum (GL+) was not required for the demonstration of inhibition.

When uncomplexed LPS was used as the inhibitor, two of the three assays (figures 3:25, 3:26, and 3:28b) produced an increased absorbance at high concentrations of inhibitor. These concentrations of LPS may permit the formation of macromolecular structures or micelles of low solubility and relatively high density which may deposit in wells causing an increased concentration of solid phase antigen, and consequently greater binding of antibody in wells. The absence of this increase in the "blank" well would, however, seem to preclude this. Another possibility is that the increase may have occurred through deposition of LPS-Ig complexes onto microtitre plates through interaction of antibodies in the complexes with solid phase antigens thereby resulting in increased concentration of both LPS and IgG in these wells, and therefore increased absorbance values.

The drop in absorbance with even higher LPS concentrations may represent an alteration of LPS towards a more soluble macromolecular form through increased association of lipid A units and thus a reduced hydrophobicity. Since this drop was also observed with OM

and bacteria as solid phase antigens, LPS at this concentration (8.0uM) may form a structure which causes LPS to be presented in a conformation as found on OM and bacteria.

There is also a possibility that the structures formed by LPS at these concentrations in this system may represent occurrences as observed in individuals with endotoxaemia. Insoluble complexes may deposit in tissues and on blood vessel walls where they could contribute to localised tissue damage as determined by Cybulsky et al (1988) and Ohshio et al (1988), while soluble forms could continue to circulate resulting in more generalised damage.

Outer membrane (OM) was highly effective at inhibiting binding of IgG to OM, uncomplexed LPS, and bacteria. Lipopolysaccharide as presented on OM is thus similar, if not identical, in antigenic presentation of LPS to bacteria. This OM fraction could thus represent a similar structure to that observed in blebs or extracellular toxic complexes (ETC) which are shed from bacteria during growth (Gankema et al 1980, Rothfield & Pearlman-Kothencz 1969; Straus 1987; Straus et al 1985; Straus et al 1988).

Little inhibition was observed in binding of IgG to LPS-polymyxin with OM, but results were highly variable and absorbances were generally higher when inhibitor (OM) was present than in absence of inhibitor (figure 3:27) while in figure 3:29c, no binding to LPS-polymyxin was obtained.

LPS-polymyxin complexes were shown in these assays (figures 3:28, and 3:29a) to possess strong inhibitory activity in binding of IgG to all antigenic preparations. Complexes in solution thus appear to represent a form of LPS which is capable of preventing binding of IgG to LPS in three other forms (uncomplexed, OM, and bacterial),

and would thus seem to present LPS in a "natural" antigenic conformation.

This inhibition system produced similar results to those from the absorption studies, although variability was seen in the stability of solid phase antigens and in the activity of each inhibitor in repeated assays. Much of the problem encountered with LPS as inhibitor could perhaps be overcome by the use of a highly soluble ionic form of lipopolysaccharide (for example the triethylamine salt form prepared after electrodialysis).

It thus appears that cross-reactivity of anti-lipopolysaccharide antibodies present in blood donor sera (and also IgGs, hyper-immune sera, or even monoclonal antibody preparations) between different preparations from the same organism, or between LPS from different organisms, can be determined by either absorption or inhibition experimentation, although refinement of techniques may be required.

The objective of this study has been fulfilled in that LPS-polymyxin complexes have been shown to represent a form of LPS which is antigenically related to that present on the bacterial surface, and therefore the CGL-pool assay with LPS-polymyxin complexes is a relevent means for the accurate detection of anti-CGL immunoglobulins in blood donor sera.

4:5. Expression of Lipopolysaccharide Epitopes on Viable Bacteria.

Many studies have been undertaken to determine the binding of anti-O-antigen and anti-CGL antibodies to viable bacteria with equivocal results (Colwell-Ward et al 1988; Crowley et al 1982; van Dijk et al 1981; Elkins & Metcalf 1985; Gigliotti & Shenep 1985; Mehta et al 1988; McCallus & Norcross 1987; Vreede et al 1986; see also section 1:3:3). In addition, a wide range of growth conditions as well as use of strains of organisms not commonly associated with septicaemia have been applied. The current study involved the use of a strain of E. coli of known clinical relevance (O18:K1 and its isogenic mutant O18:K$^-$) grown in a range of media for determination of alteration of LPS composition (analysed by PAGE) and binding of anti-CGL and an anti-O-antigen monoclonal antibodies (determined by flow cytometry).

a) An initial quantitative assessment of the proteinase K method for analysing LPS was made by determining the effect of variation of density of bacterial suspension upon the intensity of staining of different LPS components as all suspensions might not be of exactly the same density for preparation of proteinase K extracts. The results (figure 3:34) showed that various optical densities from 0.54 up to 0.64 produced little difference in staining intensities even when two different volumes of sample (10ul or 20ul) were loaded onto polyacrylamide gels. This means, therefore, that any differences observed in staining intensity occur as a result of alteration of LPS rather than through differences in the density of bacterial suspension used to prepare lipopolysaccharide samples.

b) The initial experiments with E. coli O18:K1 and O18:K$^-$ indicated that the twelve media produced little alteration in expression of

high molecular weight components of LPS. Bands of medium molecular weight showed increased staining for the non-capsulate strain, with the increase occurring stepwise with increasing concentration of serum. Growth of the capsulate strain in 100% serum produced heavier staining of core epitopes, while O18:K^- produced uniform staining of core under all conditions.

These results appear to reflect the differing requirements and different virulence factors of these two strains of E. coli. Overnight culture of the non-capsulate strain in serum induced greater expression of medium molecular weight components while O18:K1 showed only greater expression of CGL components. This could reflect greater expression of capsular material by O18:K1 enabling survival of partially R-type bacteria when grown in serum, whereas non-capsulate variants of the same strain (O18:K^-) produce greater substitution of O-antigen units onto CGL.

In addition to these alterations, a minor fast-migrating band appeared when either strain was incubated in nutrient broth or mixtures of broth and serum. This would appear to represent either a by-product of growth or possibly the presence of small peptide fragments produced during proteinase k digestion.

c) Growth of E. coli O18:K^- in nutrient broth produced greater substitution of O-antigen onto core and also increased expression of naked CGL (as represented by increased staining density of high and low molecular weight bands) throughout the logarithmic phase of growth. This pattern of staining occurred early in the growth phase, and appears to indicate a move from organisms containing a wide range of O-antigen substituents to organisms containing either very long O-antigen units or one or no O-antigen units at the expense of

medium length O-antigen chains. The presence of the fast-migrating band which appeared previously (see above) was noted after 90 min incubation.

d) The next stage involved assessment of expression of LPS epitopes on bacteria grown under different nutrient conditions. Growth kinetics of E. coli O18:K^- were firstly determined in six media. Most rapid growth was obtained in nitrogen deficient medium (see figure 3:35) and in nutrient broth, although growth in broth slowed after 150 min. The minimal medium (MALKA) also produced rapid growth, but a longer lag phase was present. The final medium (heat-inactivated sheep serum - HSS) produced the lowest growth rate.

Reduction of magnesium concentration in MALKA (see section 3:5:5) produced a marked reduction in the rate of division of bacteria. Lag phases were very long (approximately 150 min for both M01 and M10), and growth occurred at a low rate after this point.

The differences in the growth kinetics and the final optical densities of bacterial suspensions appear to rely on the availability and accessibility of carbon sources as well as the extent of limitation of cations in the media. The results obtained with magnesium limitation indicate that E. coli requires to adjust its metabolism to the low Mg^{2+} concentrations before it can divide, and the slow rate of growth achieved suggests that the enzyme systems involved in metabolism operate less efficiently under limitation of this cation (reviewed by Brown & Williams 1985).

The importance of magnesium in the replicatory processes of E. coli implied above reflects the previously reported importance of this ion (as well as other cations) in the metabolism and virulence of

Gram-negative bacteria (see for example Brown & Williams 1985).
e) It was decided that samples removed hourly for a period of 6h would be appropriate for further analysis of structural and antigenic expression of LPS. As indicated in section 3:5:6, E. coli O18:K$^-$ was grown under three conditions (NB, MALKA, and HSS). Differences were obtained in expression of core epitopes as growth in NB and MALKA produced a strongly staining R-LPS/SR-LPS region (figure 3:38a&b) while culture in HSS produced a more discrete core band (figure 3:38c). Little alteration occurred in expression of other LPS bands in organisms grown in MALKA, but when grown in NB and HSS staining of O-antigen units became heavier as time progressed. In addition, growth in HSS induced formation of a very high molecular weight component - this is possibly a form of capsular material as it has been determined that this "non-capsulate" isogenic mutant of of E. coli O18:K1 reacts with an anti-sialic acid (the component of K1 capsule) monoclonal antibody (A. Bathgate, Honours degree thesis 1988). These responses in LPS once again reflect the selective pressures upon the organisms under different growth conditions.
Determination of the binding of monoclonal antibodies to core and O-antigen (see MATERIALS AND METHODS for description), assessed through fluorescent labelling and flow cytometry (table 3:4), indicated that organisms grown in NB or MALKA showed very high reactivity with SZ184/2.5.5 (anti-O-antigen), but only negligible binding with anti-core monoclonal antibody (SZ27/150.3). Similar results were obtained with bacteria cultured overnight in magnesium limiting conditions (table 3:5). These results tend to indicate that culture of this organism in these media permits greater substitution

of O-antigen onto core units (although only NB produced greater staining of O-antigen), thereby preventing access of monoclonal antibodies to core epitopes.

When cultured in HSS, markedly different results were obtained for all samples removed during active growth of bacteria. Binding of anti-core monoclonal antibody was seen to occur at almost equivalent level to that of anti-O-antigen monoclonal antibody. Growth of E. coli O18:K^- in HSS thus alters the antigenic expression of lipopolysaccharide to permit binding of a monoclonal antibody to the core despite the increased expression of high molecular weight LPS components observed in the silver stained PAG (figure 3:38c). After overnight culture there was a marked reduction in percentage of bacteria binding to the anti-core monoclonal antibody, therefore alteration of antigenic expression terminates between 6h and 24h or culture.

f) The effect of growth of bacteria in serum was extended by comparison of capsulate and non-capsulate strains of E. coli O18 grown in untreated and in heat-inactivated sheep serum (SS and HSS respectively).

The growth kinetics (figure 3:39) produced markedly differing results. Both organisms when grown in HSS showed rapid division and increased expression of both core and O-antigen (figure 3:40). When grown in SS differences between bacteria were more obvious as O18:K1 grew at a slower rate than in HSS (possibly through alteration of virulence factors resulting from the presence of complement which possesses bacteriolytic activity against Gram-negative bacteria) while the non-capsulate strain showed a reduction in density until 240min (through the direct activation of complement by

lipopolysaccharide resulting in bacteriolysis or through antibody dependent lysis by complement) after which point division was rapid, probably through selection of serum resistant variants. Both strains when grown in SS showed increased substitution of O-antigen units onto core-glycolipid therefore showing selection of factors which could increase resistance to the bactericidal activity of serum (Goldman et al 1984; Grossman et al 1987; Porat et al 1987; Taylor & Robinson 1980; Tomas et al 1988).

Growth of E. coli O18:K$^-$ in SS and HSS followed by reaction with monoclonal antibodies produced results (table 3:6) similar to those obtained previously for growth of this organisms in HSS showing that CGL epitopes are accessible in this organism despite increased expression of O-antigen.

E. coli O18:K1 produced different results for binding of monoclonal antibodies (table 3:6) for growth in SS and HSS. In HSS binding to O-antigen occurred at high levels, but to core at only very low levels, while in SS, binding to both core and O-antigen were fairly high during the early log phase, but fell fell to very low levels over the course of growth.

As growth in SS progresses, however, it appears that the capsule is altered in some way (perhaps through increase in density) and access of the monoclonal antibodies to LPS epitopes is restricted despite increased expression of O-antigen as shown by silver staining. A similar alteration of capsular structure could therefore occur in vivo and could thus affect bacterial virulence.

These results appear to indicate that the presence of capsule does not preclude the binding of anti-lipopolysaccharide antibodies. This result is supportive of the data described by Williams et al (1988)

who produced a similar finding with K. aerogenes in a different system, and also those of Kaufman et al 1986 who showed that an anti-O-antigen antibody could protect mice against lethal challenge with the capsulate organism E. coli O18:K1. Pluschke & Achtman (1985) have also achieved protection against capsulate organisms by use of an anti-O-antigen monoclonal antibody, therefore access must be gained to O-antigen in both of these in vivo systems. Also indicated by these results is the ability of anti-core monoclonal antibodies to bind to viable organisms under certain culture conditions, thereby firmly supporting the argument in support of the ability of anti-CGL to bind to intact organisms (McCallus & Norcross 1987; Williams et al 1988) although the results obtained with the capsulate strain indicate that anti-CGL may not be opsonic (as has already been observed by Gigliotti & Shenep 1985; Mehta et al 1988; Vreede et al 1986) - but they may be able to gain access to epitopes. This is particularly relevant in vivo where mainly capsulate strains are encountered, and where the precise phase of growth and expression of virulence factors remains a matter of conjecture.

g) Because of the strong binding of the anti-core monoclonal antibody under a variety of conditions (even when increased substitution of O-antigen was evident) it was decided to determine whether binding of natural sheep antibodies which recognise the O18 O-antigen were permitting access of anti-CGL as postulated by Frank et al (1987). To carry this out, untreated serum was absorbed with either E. coli O18:K$^-$ or the organism with the unrelated O-antigen E. coli O86:K61 (see section 4:3 for antigenic relationship). Bacteria (E. coli O18:K1 or O18:K$^-$) were then cultured in the two

absorbed sera and in untreated serum. The results produced (table 3:9) were at variance with those obtained previously, as both organisms grown in untreated serum showed significant binding with only O-antigen and not CGL-reactive monoclonal antibody. No reduction was observed in reactivity with anti-O-antigen monoclonal antibody for O18:K1 as seen above, and binding to core was insignificant. This may have occurred as a result of the inoculum being prepared from a culture which itself had undergone three subculture processes instead of the single subculture step usually applied. Repeated subculture may therefore have selected particular variants which differ from the initial population in the inoculum in LPS antigenicity.

Comparison can, however, still be made between the organisms grown in serum which was absorbed with either O18- or O86-containing strains. The results were similar for both absorptions, with O18:K1 showing no binding and minimal binding to core and O-antigen respectively, and O18:K^- showing moderate to high binding to only O-antigen specific monoclonal antibody. Because of the low binding of the anti-CGL monoclonal antibody in bacteria grown in SS, it cannot be determined whether natural sheep antibodies to O-antigen are enabling binding of anti-CGL. There does, however, appear to exist some selective pressure on the capsulate strain grown in absorbed serum for restriction of access of O-antigen specific antibodies.

The labelling of bacteria with antibodies conjugated to FITC followed by analysis of binding characteristics by flow cytometry provides a highly sensitive technique for determination of binding of monoclonal antibodies to lipopolysaccharide and could be applied

to other bacterial cell surface components. There were, however, several points at which anomalous results were obtained, and several instances of test values below control values (bacteria reacted only with anti-mouse-FITC labelled antibody) occurred. In the main, these values were less than 5% below control values, but negative values of up to 35% occurred on a few occasions. This indicates that this assay requires refinement to determine optimal concentrations of bacteria, monoclonal antibodies and secondary antibody as well as incubation times and cytometry parameters.

Despite the above problems it has still been possible to determine in conjunction with proteinase K digestion of bacteria and polyacrylamide gel electrophoresis the following points:

i) expression of lipopolysaccharide epitopes on the cell surface can be markedly altered by variation of the growth environment,

ii) accessibility of anti-CGL is restricted to certain growth conditions and care must therefore be taken in assessment of binding and opsonic ability of antibodies with respect to culture conditions and phase of growth,

and iii) capsular material has an important bearing on the ability of antibodies to bind to O-antigen and CGL in organisms grown in untreated serum which therefore implies that therapeutic preparations should contain both bacteriolytic (anti-O-antigen and capsule) and anti-toxic (anti-CGL) antibodies to permit effective treatment of septicaemia.

4:6. In vitro Anti-Endotoxic Activity of Human Anti-Lipopolysaccharide Immunoglobulins.

The inhibitory activity of human immunoglobulins on the activation of *Limulus* amoebocyte lysate (LAL) by purified lipopolysaccharides was determined as an indication of the potential of antibodies to neutralise the toxic activities of LPS.

a) Firstly the extent of activation of LAL was determined and the activities of the LPSs assessed in descending order from most active by weight was:

1. *S. typhimurium* R1102, Re
   S. minnesota R595, Re
2. *E. coli* O18
   " " " O16
   " " " O6
3. *P. aeruginosa* Habs type 1
4. *S. typhimurium* R878, Rc
5. " " " R1542, Ra
6. *E. coli* R2, Ra
7. " " " J5, Rc

These results are in general agreement with those of Cohen & McConnell (1984), Guyomard & Darbord (1985) and Warren *et al* (1987) in their assessment of the activity of LPSs in a LAL assay. The present assay was carried out several times to determine the activities of all lipopolysaccharides, with LPS from *E. coli* O18 common to all repetitions. The activities of O18 LPS in each assay was plotted on one graph (figure 3:42) and indicated that test-to-test variation did occur, but that each activation curve was

very similar in both shape and absorbance values at each LPS concentration. This indicated that this assay was reproducible and accurate.

b) Inhibiton was firstly attempted with human serum to determine whether inhibitory activity would be demonstrable in the assay system used (see MATERIALS AND METHODS). As shown in figure 3:43, two normal human sera, with high (GL+) and low (GL-) anti-CGL completely removed the LAL activity of E. coli 018 LPS. Because of the differences in levels of anti-CGL (assigned values of 100% for GL+ and 10% for GL-) the inhibitory activity when present undiluted would appear to reside in factors other than or additional to immunoglobulins of class G. It is possible that IgM may have contributed to this activity, but as IgM levels were not determined little can be inferred. Another factor which has been implicated as a modulator of endotoxin activity in a LAL assay system is high-density lipoprotein - HDLP (Warren et al 1987), although other serum factors may also contribute (see Berger & Beger 1988 for short review).

Irrespective of the factor responsible, it was clearly demonstrated that LAL activity of purified LPS could be inhibited in this assay system, therefore assessment of the capacity of purified IgGs was carried out.

c) Initial results indicated that IgGs themselves possessed extremely high LAL activity (figure 3:44a). A series of studies indicated that, firstly, digestion of IgG with proteinase K followed by PAGE and silver staining for LPS did not reveal the presence of any pattern consistent with either rough or smooth type LPS, and secondly, that the LAL activity of IgG was not inhibitable by

polymyxin B even at concentrations which would neutralise an amount of LPS of equivalent LAL activity. It was, however, not surprising in retrospect that LPS was not detectable by silver staining as the maximum equivalent concentration of LPS in the IgG of highest LAL activity was approximately 167pg/ml (approximately 2000EU/ml, where 12EU is equivalent to 1.0pg of LPS per ml), therefore from the volume loaded onto PAG (50ul) only 8.35ng of LPS could be present. The approximate amount loaded onto gels from normal poteinase K digests is 10ng (where a bacterial suspension with an A_{525} of 0.5-0.6 contains approximately 10^9organisms/ml and an organism contains femtogram - 10^{-15}g - amounts of LPS). The second line of evidence does produce a strong indication that the activity of the IgGs in the LAL assay is caused by factors other than contaminating LPS. Possible candidates include a range of polysaccharide and protein compounds (summarised by Baek _et al_ 1985, Berger & Beger 1988, and Berger _et al_ 1988) which are known to possess some LAL activating capacity. It is possilble that some of these activators may have eluted during column chromatography for the purification of IgGs (see MATERIALS AND METHODS). This activity was greatly reduced through dilution by a factor of 625.

d) Inhibition was carried out with IgG at a dilution pf 1:500 as dilution by 1:1000 was shown to possess only minimal inhibitory activities (see figure 3:47). This dilution produced residual LAL activity of IgGs, but inhibition was detectable in many instances. IgG and LPS were incubated prior to addition of LAL to permit maximal binding of anti-CGL (which may be present at only low levels and which may possess only low avidity for CGL epitopes as suggested by Appelmelk _et al_ 1986).

A high degree of variability was present between successive measurements of inhibition despite identical conditions and in contrast with the good reproducibility in measurement of LPS. This may have arisen through differing solubilities of LPS in the presence of IgG molecules, or as a result of the presence of different cations in IgG and LPS preparations. Different species of cations have been shown to alter the solubility of LPS (Baggerman et al 1988; Brade et al 1987a; Galanos & Luderitz 1975; Galanos & Luderitz 1976) and also to affect the activity of LAL enzymes responsible for activation, and it is therefore possible that this factor may contribute to the variability observed. Another possibility that exists is in the pre-incubation step of IgG and LPS which was carried out at room temperature. It is possible that day-to-day variation may have altered the interaction between LPS and IgG, therefore accounting for the variability.

One further possibility which should be investigated is the LAL activity of outer membrane vesicles. These may be representative of 'extracellular toxic complexes' which have been detected in vivo (Straus 1987; Straus et al 1985; Straus et al 1988) and which contribute to the manifestations of Gram-negative bacterial infection. In addition to LPS and phospholipid, these contain proteins which may also contribute to toxicity (Bjornson et al 1988; Johns et al 1988). This therefore represents one direction which could be followed in determination of toxicity and of the anti-endotoxic activity of immunoglobulin preparations, and may lead to clarification of the role of immunoglobulins in neutralisation of endotoxic activity.

It is thus suggested that a standard salt form of

lipopolysaccharides of high solubility (prepared after electrodialysis of LPS - see Galanos & Luderitz 1975) is used, and that IgG preparations are extensively purified to remove contaminants such as dextran or LPS to enable accurate determination of anti-endotoxic activity of immunoglobulins.

Many inhibitions with IgG did, though, produce some inhibitory activity. Inhibitory capacity did not absolutely reflect the antibody profiles of the IgGs (see figure 3:4) particularly with regard to *E. coli* 06 LPS which showed only minimal inhibition with any IgG despite the presence of specific antibody. Additionally, some LPS showed increased activity in the presence of certain IgGs - possibly through the additive effect of the LAL activities of IgG and LPS, although there is the possibility that binding of LPS by IgG solubilised the LPS thereby exposing more lipid A sites which could activate LAL enzymes.

d) Polymyxin was shown to possess strongly inhibitory activity against all 4 LPS assessed, indicating that polymyxin binds to the site responsible for LAL activation, that is the lipid A. This polycationic antimicrobial agent possessed greater inhibitory activity than any of the IgGs.

This series of experiments demonstrated that IgG prepared from human serum may possess anti-endotoxic activity, but refinement of this assay system is required to produce definitive indications of this activity. If, however, this assay can be shown to produce consistent inhibition it may find a strong relationship to the activity of anti-endotoxin antibodies *in vivo*.

4:7. Protective Activities of Immunoglobulins in vivo.

The serotypes of organism chosen to challenge animal models have been diverse, but Escherichia coli and Pseudomonas aeruginosa have been used most frequently. These two organisms represent the species which contribute the highest proportion of fatalities from Gram-negative septicaemia and are therefore of great relevance in assessment of septicaemia in animals. The strains of E. coli used in this study have all been relevant to septicaemia as determined by Cross et al (1983) and Cheasty et al (1977), but many other studies of the protective activity of various immunoglobulin preparations have been carried out with serotypes of bacteria not commonly associated with septicaemia. It is therefore hoped that a closer reflection of human septicaemia is obtained by application of the relevant organisms as in this assessment.

Many protection studies also involve administration of antibody many hours prior to challenge with LPS or bacteria. This obviously does not follow the situation in patients where signs and symptoms of septicaemia are observed prior to initiation of therapy. It may thus be more appropriate for therapy in models to be instigated after challenge with bacteria or LPS, and this was indeed the preferred timing of antibody administration in the current assessment.

Despite the above points of contention with the clinical situation, it is beyond doubt that challenge of animals with LPS or bacteria does give an indication of the action of these agents upon an organism. Application of therapeutic agents (before, after, or simultaneously with challenge) can allow assessment of their potential to protect against the effects of Gram-negative bacterial and lipopolysaccharide challenge, and the results obtained in this

study are assessed below.

a) All experiments indicated that mice are particularly refractory to the lethal effects of Gram-negative bacterial or lipopolysaccharide challenge. One means of compromise (co-inoculation of bacteria with mucin and haemoglobin) produced a large reduction in the lethal dose of bacteria, in particular the capsulate E. coli O18:K1.

b) Clear differences were obtained in the lethal doses of smooth and rough strains of P. aeruginosa and E. coli (tables 3:8 and 3:11 respectively), confirming the importance of the presence of O-antigen in the virulence of Gram-negative bacteria.

c) The culture of P. aeruginosa Habs type 1 in two different media (nutrient broth and MALKA) indicated a small increase in lethality of bacteria grown in the minimal medium. These differences may have been reflected in alteration of LPS structure, but at the time this was not assessed. This alteration in virulence supports the data presented in section 3:5 showing that alteration of growth medium causes changes in surface components of bacteria which in turn may affect the virulence of the bacterium.

d) A normal human serum containing high levels of anti-CGL (HI-NS - see table 3:11) was shown to possess limited protective action against lethal challenge with P. aeruginosa Habs type 1 and several O-serotypes of E. coli which are predominant in septicaemia.

e) Use of mucin and haemoglobin to lower the lethal dose of bacteria failed to produce any noticeable protection with 4 normal sera (GL+, GL-, MED1, and MED2 - see table 3:14) against E. coli O18:K1 despite a challenge dose of only 60 organisms (5 times minimum dose required to result in death of all mice challenged). The results presented in

table 3:16 did, however, show that some protective activity was obtained in an adaptation of this model (where mice were challenged with lower numbers of bacteria) with two IgG preparations containing high levels of anti-CGL (IgGs 5 and 24 - see figure 3:16 for antibody profiles) and a monoclonal antibody specific for the O-antigen of this organism (SZ184/2.5.5 - see MATERIALS AND METHODS).

The very low lethal dose of bacteria in the mucin/haemoglobin model of sepsis may partly be due to the virulence factors of the above organisms, but it does not appear that this model provides an appropriate means for the measurement of protection because of the severe increase in virulence of bacteria in this model. Both factors used to increase the virulence can be dealt with separately:

1. Haemoglobin provides a source of iron (see section 1:4) to enable bacteria to multiply rapidly in an otherwise iron-depleted environment. Iron is known to be an essential element in bacterial metabolism and its presence may permit alteration of virulence factors (including LPS, outer membrane proteins and capsule) and thus the protective capacity of immunoglobulins (see, for example, Brown & Williams 1985).

2. Mucin would appear to provide a highly protective environment similar to that present in the slime excretions produced by some bacteria during infection, notably _P. aeruginosa_ (Pollack 1984) among Gram-negative bacteria. Because of the presence of iron, however, bacteria can multiply rapidly in the absence of any host immune response. During growth in this environment, bacteria may be producing the extracellular toxic complexes (ETC) as encountered in _K. aerogenes_ (Straus 1987) and _P. cepacia_ (Straus _et al_ 1988)

infections, thereby resulting in death. A further possibility is that bacteria are released *en masse* once the replication process has enabled bacteria to occupy the entire volume of mucin, resulting in a massive efflux of bacteria into the peritoneum and effectively producing a large bacterial inoculum.

f) One further compromised model of bacterial challenge was applied. Sub-lethal neutropenia was induced with cyclophosphamide in mice (see MATERIALS AND METHODS), and mice were challenged with bacteria and haematin (as a source of iron) followed by administration of immunoglobulins. The overall results indicated that noticeable but insignificant protection was demonstrated by three of the five purified human IgG preparations (figure 3:17). These activities did not, however, reflect the extent of cross-reactive IgG in the preparations, although the presence of different subclasses of IgG may have an important bearing on the outcome. This model was representative of many patients who develop septicaemia through the presence of neutropenia (see section 1:1:2). Despite induction of neutropenia and the presence of excess iron, the lethal dose was still very high (LD_{50} in the range of 10^6-10^7 organisms), but the model has still provided a slight indication of the potential protective capacity of IgG purified from human blood donors. Further assessment of the extent of neutropenia (as carried out by Vuopio-Varkila, 1988) and also the contribution of the iron in haematin appears to be necessary to refine this model.

g) The toxicity of purified lipopolysaccharide in mice was shown to be markedly reduced through co-inoculation with **D**-galactosamine and, in agreement with the literature (Galanos *et al* 1979), a reduction of approximately 10^5-fold was obtained in lethal dose. The choice of

solvent was also shown to have a bearing on the lethal activity of LPS (section 3:7:4), thus stressing the importance of the salt form of LPS on the toxic activity of LPS as first determined by Galanos and colleagues (Galanos & Luderitz 1975; Galanos & Luderitz 1976). Application of immunoglobulin to mice challenged with lethal doses of LPS produced only small protective activity as shown in section 3:7:4, and results were highly variable from test to test. Assessment of the protective activity of immunoglobulins was thus more readily demonstrated in bacterial challenge models.

Careful consideration must therefore be taken in development of animal models of septicaemia. Particular regard must be given to the virulence factors possessed by organisms in relation to known septicaemia strains, the culture conditions imposed for preparation of challenge inocula, and in the mode of compromise induced in these animals.

Further analysis of the immunoglobulin preparations may also be necessary. It has been determined (Nys et al 1988) that the development of anti-LPS of different subclasses has an important bearing upon the outcome of septicaemia in a patient, and therefore subclass reactivity with CGL epitopes may have a bearing on the potential therapeutic value of IgG preparations for the treatment of septicaemia.

From the above presentation, it would thus appear that IgG prepared from the serum of blood donors possessing high levels of anti-CGL may provide a suitable means of prevention of fatalitites arising from systemic Gram-negative bacterial infection in human patients

although the efficacy of these human immunoglobulin preparations remains to be fully examined in animal models.

Summary of Conclusions.

In view of the results obtained during the course of this investigation of some activities of anti-lipopolysaccharide immunoglobulins, the following conclusions can be made:

The LPS-polymyxin ELISA for detection of anti-CGL immunoglobulins represents an accurate and reproducible assay which identifies antibodies of appropriate specificity for therapeutic application. This arises as the antigenic presentation of LPS appears to resemble other LPS-containing antigens. The clinical data presented here indicated that the choice of antigens has been appropriate in relation to the antibodies observed to be most closely related to the presence or absence of endotoxin in shock patients. It would thus appear that use of this assay may result in the selection of an intravenous immunoglobulin preparation of high therapeutic potential.

The antigenic relationships between various LPS molecules has been extended, and implications in the choice of antigens for assays and for production of antibodies has been noted.

Alteration of bacterial lipopolysaccharide for bacteria grown under different media conditions has indicated that care must be taken in extrapolating information obtained from *in vitro* determinations of anti-LPS antibody activities. As a result of the differential binding of antibodies to core and O-antigen for bacteria grown under different nutrient conditions and because of the influence of capsular material of this activity, bacterial strains and growth conditions must be chosen carefully to mimic *in vivo* conditions.

Some anti-bacterial and anti-endotoxin activity was demonstrable in a range of *in vitro* and *in vivo* models with IgG purified from human

blood donors. It therefore appears that selection of donors with high-titres of anti-CGL antibodies can provide a suitable source of antibodies for passive immunisation of patients with systemic Gram-negative bacterial infection and septic shock in conjunction with currently available supportive and antimicrobial agent therapy.

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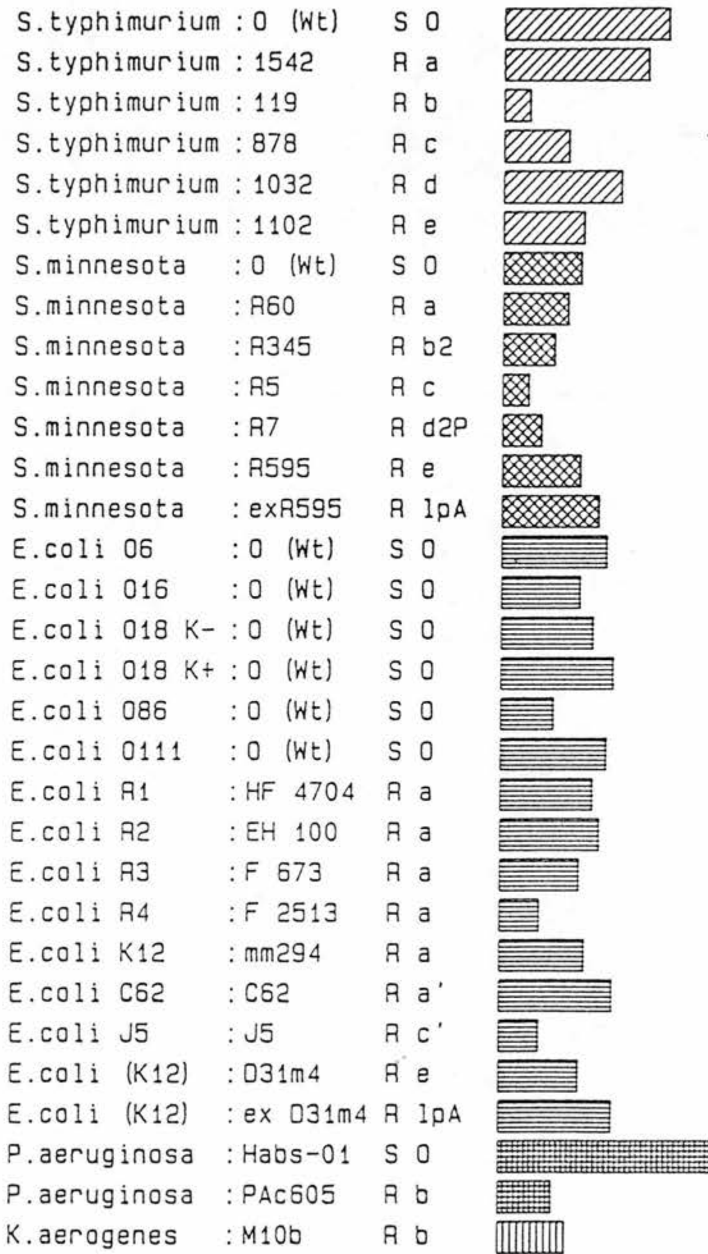
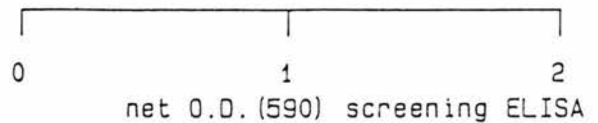
APPENDIX 1

IgG-01

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

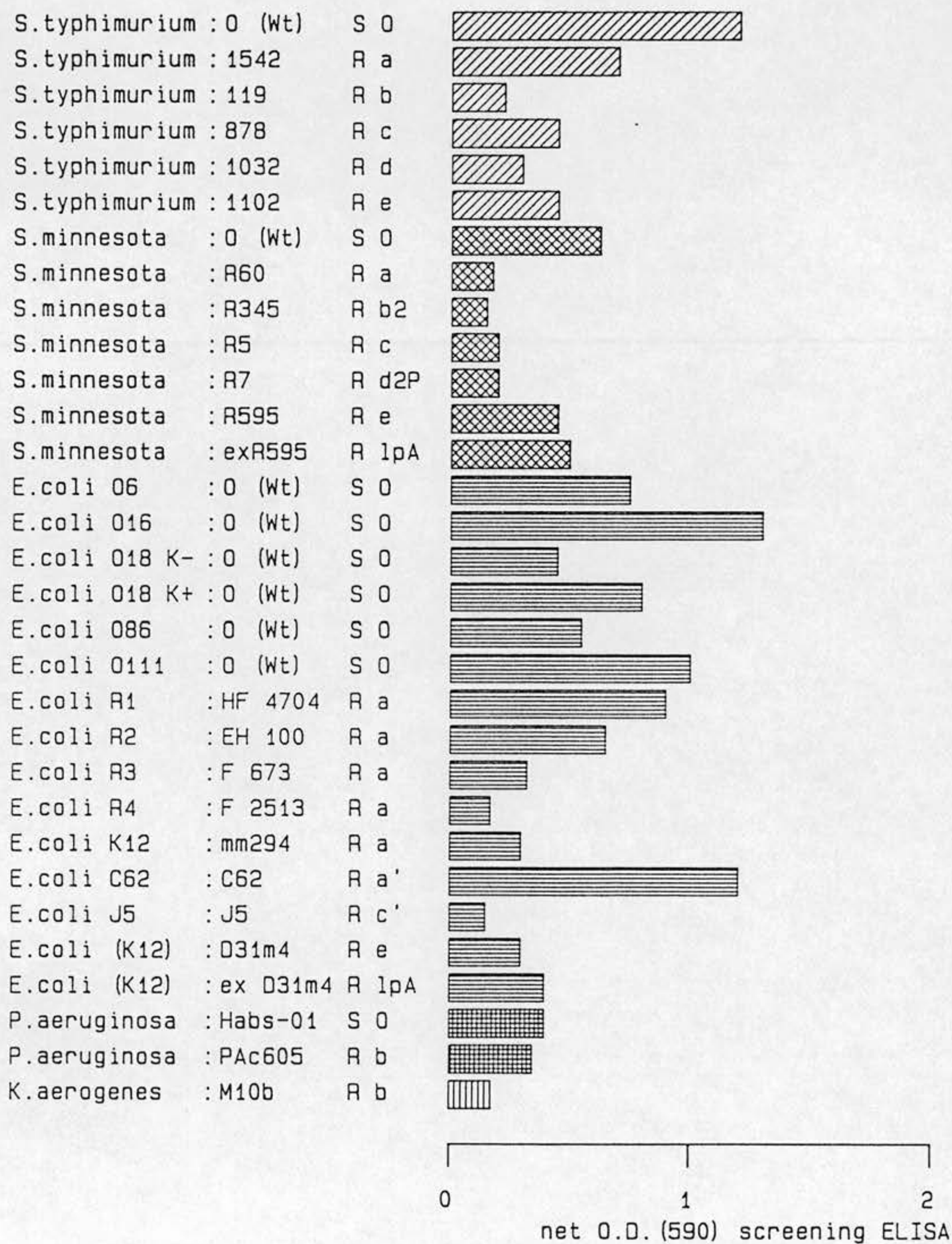




IgG-02

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)



IgG-03

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Strain		Type
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-04

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Organism	Strain	Type
S.typhimurium	0 (Wt)	S 0
S.typhimurium	1542	R a
S.typhimurium	119	R b
S.typhimurium	878	R c
S.typhimurium	1032	R d
S.typhimurium	1102	R e
S.minnesota	0 (Wt)	S 0
S.minnesota	R60	R a
S.minnesota	R345	R b2
S.minnesota	R5	R c
S.minnesota	R7	R d2P
S.minnesota	R595	R e
S.minnesota	exR595	R lpA
E.coli 06	0 (Wt)	S 0
E.coli 016	0 (Wt)	S 0
E.coli 018 K-	0 (Wt)	S 0
E.coli 018 K+	0 (Wt)	S 0
E.coli 086	0 (Wt)	S 0
E.coli 0111	0 (Wt)	S 0
E.coli R1	HF 4704	R a
E.coli R2	EH 100	R a
E.coli R3	F 673	R a
E.coli R4	F 2513	R a
E.coli K12	mm294	R a
E.coli C62	C62	R a'
E.coli J5	J5	R c'
E.coli (K12)	D31m4	R e
E.coli (K12)	ex D31m4	R lpA
P.aeruginosa	Habs-01	S 0
P.aeruginosa	PAc605	R b
K.aerogenes	M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-05

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Strain	Antigen	Type
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-06

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Organism	Strain	Chemotype
S.typhimurium	: O (Wt)	S O
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: O (Wt)	S O
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli O6	: O (Wt)	S O
E.coli O16	: O (Wt)	S O
E.coli O18 K-	: O (Wt)	S O
E.coli O18 K+	: O (Wt)	S O
E.coli O86	: O (Wt)	S O
E.coli O111	: O (Wt)	S O
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S O
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-07

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Species	Strain	Type
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

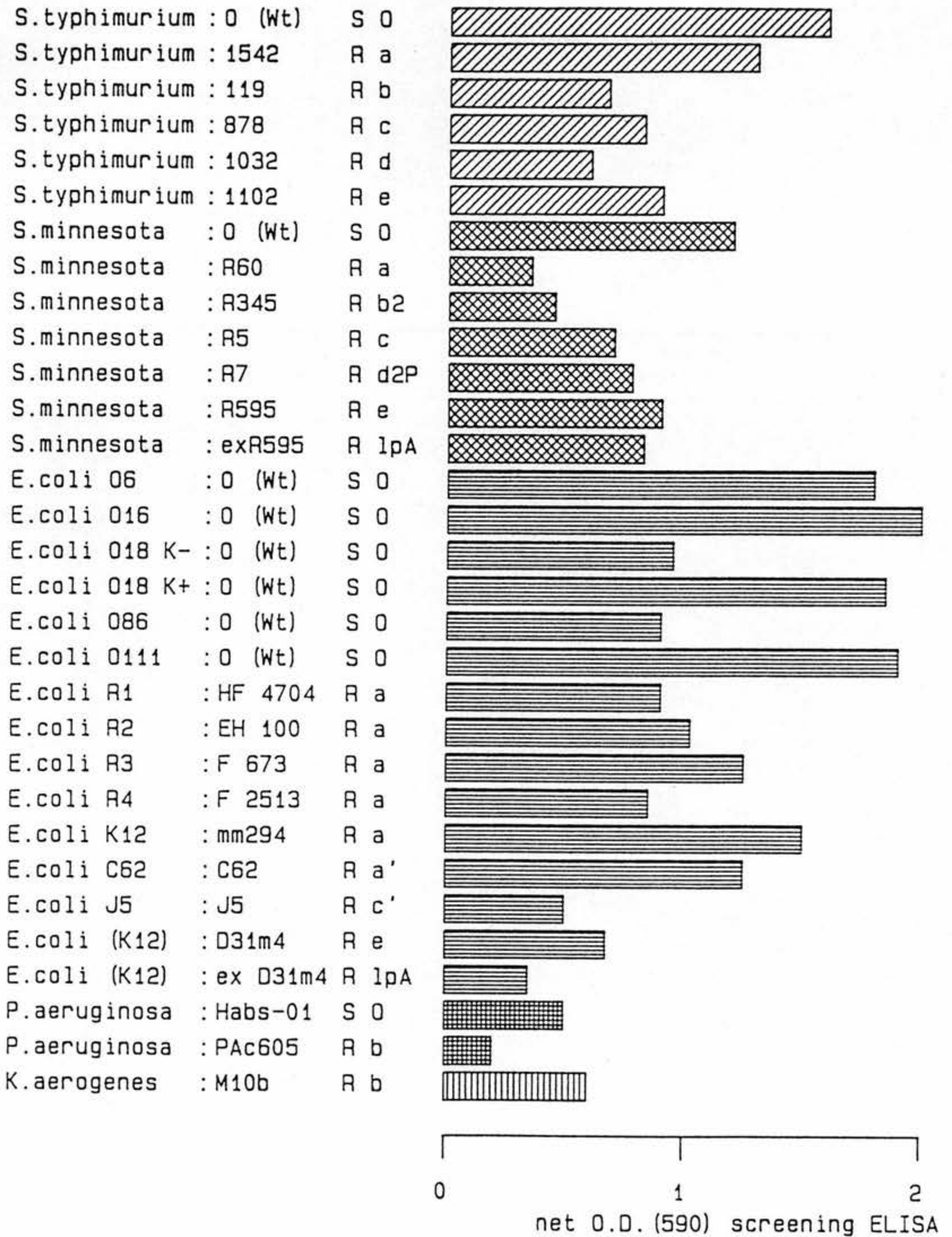
0 1 2

net O.D. (590) screening ELISA

IgG-08

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)



IgG-09

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-10

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Antigen	Strain	Type
S.typhimurium	0 (Wt)	S 0
S.typhimurium	1542	R a
S.typhimurium	119	R b
S.typhimurium	878	R c
S.typhimurium	1032	R d
S.typhimurium	1102	R e
S.minnesota	0 (Wt)	S 0
S.minnesota	R60	R a
S.minnesota	R345	R b2
S.minnesota	R5	R c
S.minnesota	R7	R d2P
S.minnesota	R595	R e
S.minnesota	exR595	R lpA
E.coli O6	0 (Wt)	S 0
E.coli O16	0 (Wt)	S 0
E.coli O18 K-	0 (Wt)	S 0
E.coli O18 K+	0 (Wt)	S 0
E.coli O86	0 (Wt)	S 0
E.coli O111	0 (Wt)	S 0
E.coli R1	HF 4704	R a
E.coli R2	EH 100	R a
E.coli R3	F 673	R a
E.coli R4	F 2513	R a
E.coli K12	mm294	R a
E.coli C62	C62	R a'
E.coli J5	J5	R c'
E.coli (K12)	D31m4	R e
E.coli (K12)	ex D31m4	R lpA
P.aeruginosa	Habs-01	S 0
P.aeruginosa	PAc605	R b
K.aerogenes	M10b	R b

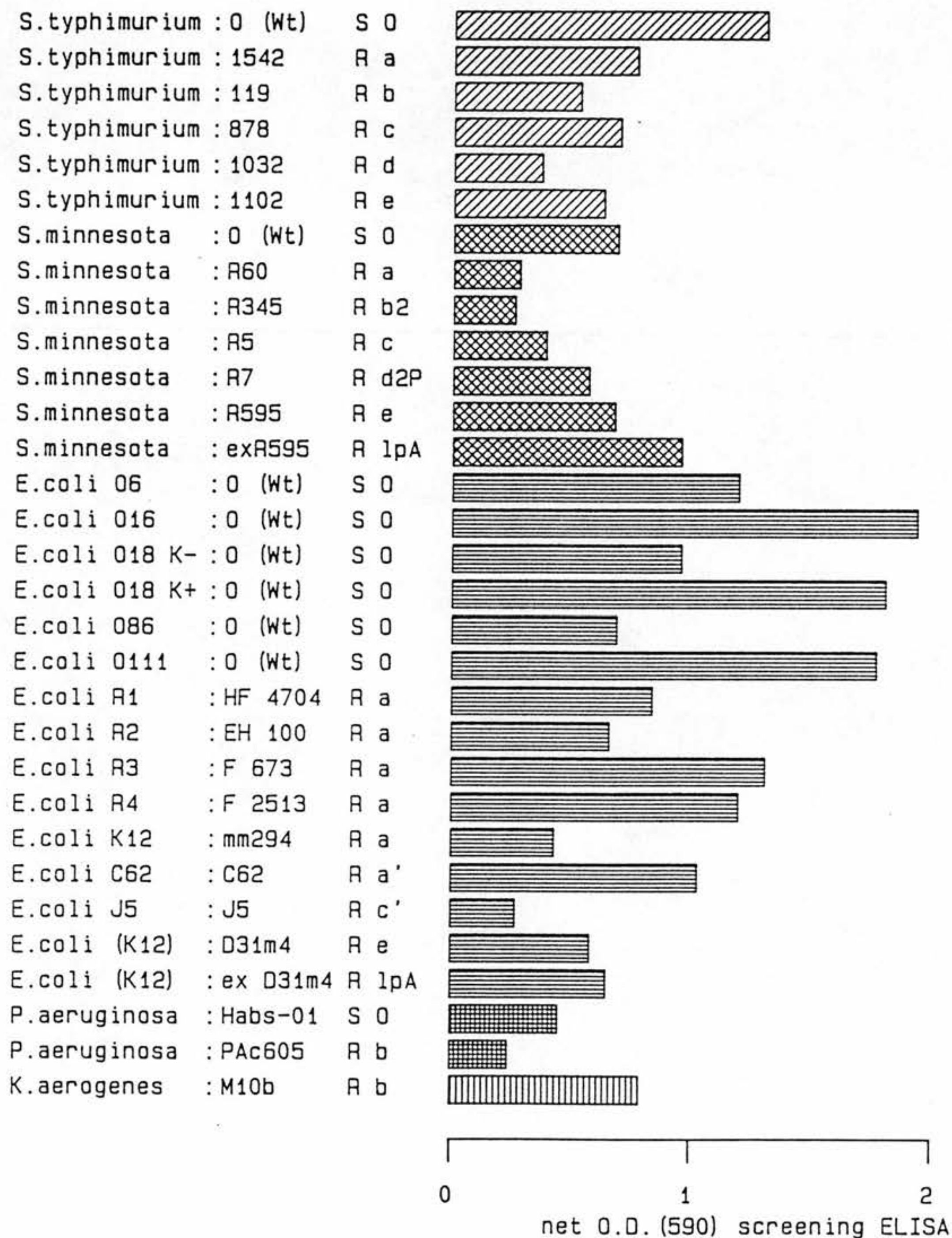
0 1 2

net O.D. (590) screening ELISA

IgG-11

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)



IgG-13

dilution - 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Strain		
S.typhimurium	: O (Wt)	S O
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: O (Wt)	S O
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli O6	: O (Wt)	S O
E.coli O16	: O (Wt)	S O
E.coli O18 K-	: O (Wt)	S O
E.coli O18 K+	: O (Wt)	S O
E.coli O86	: O (Wt)	S O
E.coli O111	: O (Wt)	S O
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S O
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-14

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

S.typhimurium : 0 (Wt) S 0
S.typhimurium : 1542 R a
S.typhimurium : 119 R b
S.typhimurium : 878 R c
S.typhimurium : 1032 R d
S.typhimurium : 1102 R e
S.minnesota : 0 (Wt) S 0
S.minnesota : R60 R a
S.minnesota : R345 R b2
S.minnesota : R5 R c
S.minnesota : R7 R d2P
S.minnesota : R595 R e
S.minnesota : exR595 R lpA
E.coli 06 : 0 (Wt) S 0
E.coli 016 : 0 (Wt) S 0
E.coli 018 K- : 0 (Wt) S 0
E.coli 018 K+ : 0 (Wt) S 0
E.coli 086 : 0 (Wt) S 0
E.coli 0111 : 0 (Wt) S 0
E.coli R1 : HF 4704 R a
E.coli R2 : EH 100 R a
E.coli R3 : F 673 R a
E.coli R4 : F 2513 R a
E.coli K12 : mm294 R a
E.coli C62 : C62 R a'
E.coli J5 : J5 R c'
E.coli (K12) : D31m4 R e
E.coli (K12) : ex D31m4 R lpA
P.aeruginosa : Habs-01 S 0
P.aeruginosa : PAc605 R b
K.aerogenes : M10b R b

0 1 2

net O.D. (590) screening ELISA

IgG-15

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Organism	Strain	Type
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-16

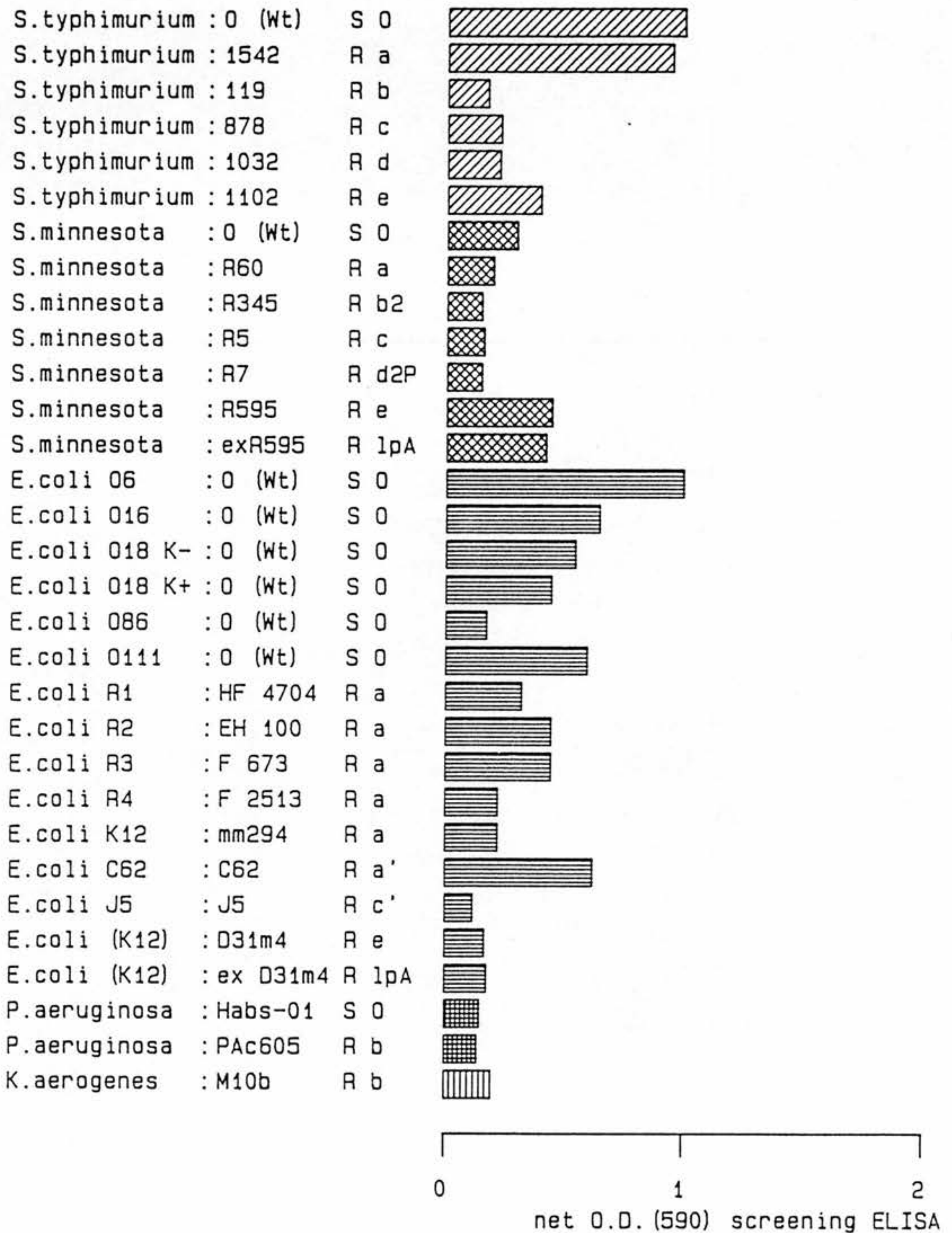
dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Strain			
S.typhimurium	: O (Wt)	S	O
S.typhimurium	: 1542	R	a
S.typhimurium	: 119	R	b
S.typhimurium	: 878	R	c
S.typhimurium	: 1032	R	d
S.typhimurium	: 1102	R	e
S.minnesota	: O (Wt)	S	O
S.minnesota	: R60	R	a
S.minnesota	: R345	R	b2
S.minnesota	: R5	R	c
S.minnesota	: R7	R	d2P
S.minnesota	: R595	R	e
S.minnesota	: exR595	R	lpA
E.coli O6	: O (Wt)	S	O
E.coli O16	: O (Wt)	S	O
E.coli O18 K-	: O (Wt)	S	O
E.coli O18 K+	: O (Wt)	S	O
E.coli O86	: O (Wt)	S	O
E.coli O111	: O (Wt)	S	O
E.coli R1	: HF 4704	R	a
E.coli R2	: EH 100	R	a
E.coli R3	: F 673	R	a
E.coli R4	: F 2513	R	a
E.coli K12	: mm294	R	a
E.coli C62	: C62	R	a'
E.coli J5	: J5	R	c'
E.coli (K12)	: D31m4	R	e
E.coli (K12)	: ex D31m4	R	lpA
P.aeruginosa	: Habs-01	S	O
P.aeruginosa	: PAc605	R	b
K.aerogenes	: M10b	R	b

0 1 2

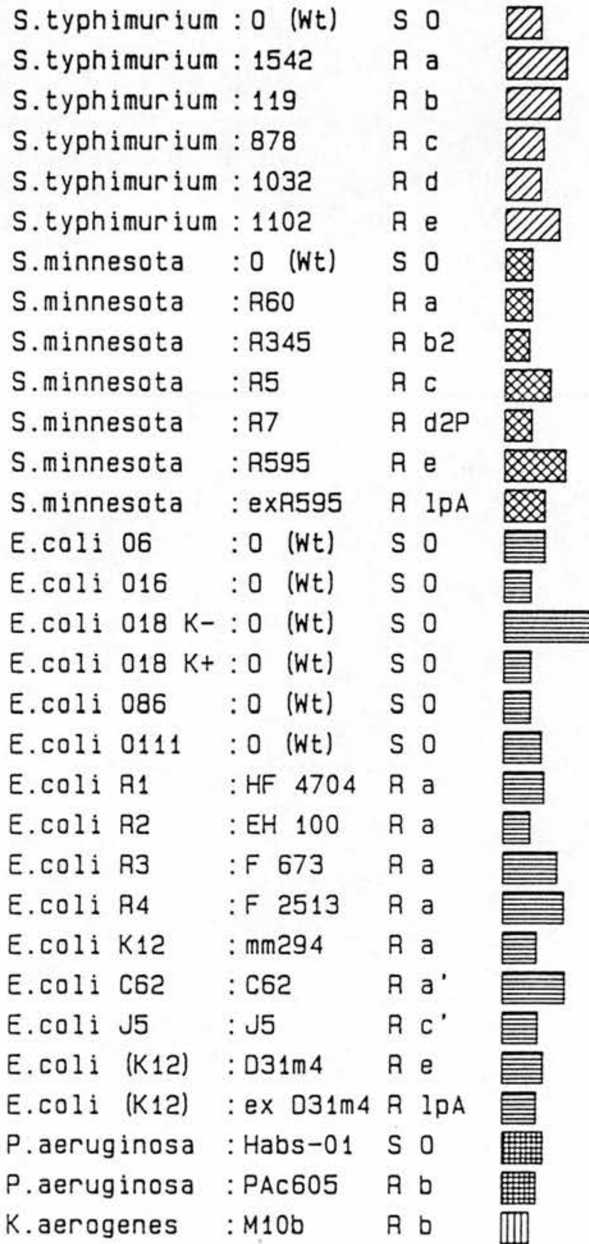
net O.D. (590) screening ELISA

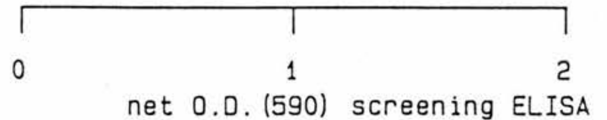


IgG-17
dilution = 1/ 100
ELISA ANTIGENS (LPS-polymyxin complexes)
S.typhimurium : 0 (Wt) S 0
S.typhimurium : 1542 R a
S.typhimurium : 119 R b
S.typhimurium : 878 R c
S.typhimurium : 1032 R d
S.typhimurium : 1102 R e
S.minnesota : 0 (Wt) S 0
S.minnesota : R60 R a
S.minnesota : R345 R b2
S.minnesota : R5 R c
S.minnesota : R7 R d2P
S.minnesota : R595 R e
S.minnesota : exR595 R lpA
E.coli 06 : 0 (Wt) S 0
E.coli 016 : 0 (Wt) S 0
E.coli 018 K- : 0 (Wt) S 0
E.coli 018 K+ : 0 (Wt) S 0
E.coli 086 : 0 (Wt) S 0
E.coli 0111 : 0 (Wt) S 0
E.coli R1 : HF 4704 R a
E.coli R2 : EH 100 R a
E.coli R3 : F 673 R a
E.coli R4 : F 2513 R a
E.coli K12 : mm294 R a
E.coli C62 : C62 R a'
E.coli J5 : J5 R c'
E.coli (K12) : D31m4 R e
E.coli (K12) : ex D31m4 R lpA
P.aeruginosa : Habs-01 S 0
P.aeruginosa : PAc605 R b
K.aerogenes : M10b R b
0
1
2
net O.D. (590) screening ELISA

IgG-18

dilution = 1/ 100





IgG-19

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Strain			
S.typhimurium	: 0 (Wt)	S	0
S.typhimurium	: 1542	R	a
S.typhimurium	: 119	R	b
S.typhimurium	: 878	R	c
S.typhimurium	: 1032	R	d
S.typhimurium	: 1102	R	e
S.minnesota	: 0 (Wt)	S	0
S.minnesota	: R60	R	a
S.minnesota	: R345	R	b2
S.minnesota	: R5	R	c
S.minnesota	: R7	R	d2P
S.minnesota	: R595	R	e
S.minnesota	: exR595	R	lpA
E.coli 06	: 0 (Wt)	S	0
E.coli 016	: 0 (Wt)	S	0
E.coli 018 K-	: 0 (Wt)	S	0
E.coli 018 K+	: 0 (Wt)	S	0
E.coli 086	: 0 (Wt)	S	0
E.coli 0111	: 0 (Wt)	S	0
E.coli R1	: HF 4704	R	a
E.coli R2	: EH 100	R	a
E.coli R3	: F 673	R	a
E.coli R4	: F 2513	R	a
E.coli K12	: mm294	R	a
E.coli C62	: C62	R	a'
E.coli J5	: J5	R	c'
E.coli (K12)	: D31m4	R	e
E.coli (K12)	: ex D31m4	R	lpA
P.aeruginosa	: Habs-01	S	0
P.aeruginosa	: PAc605	R	b
K.aerogenes	: M10b	R	b

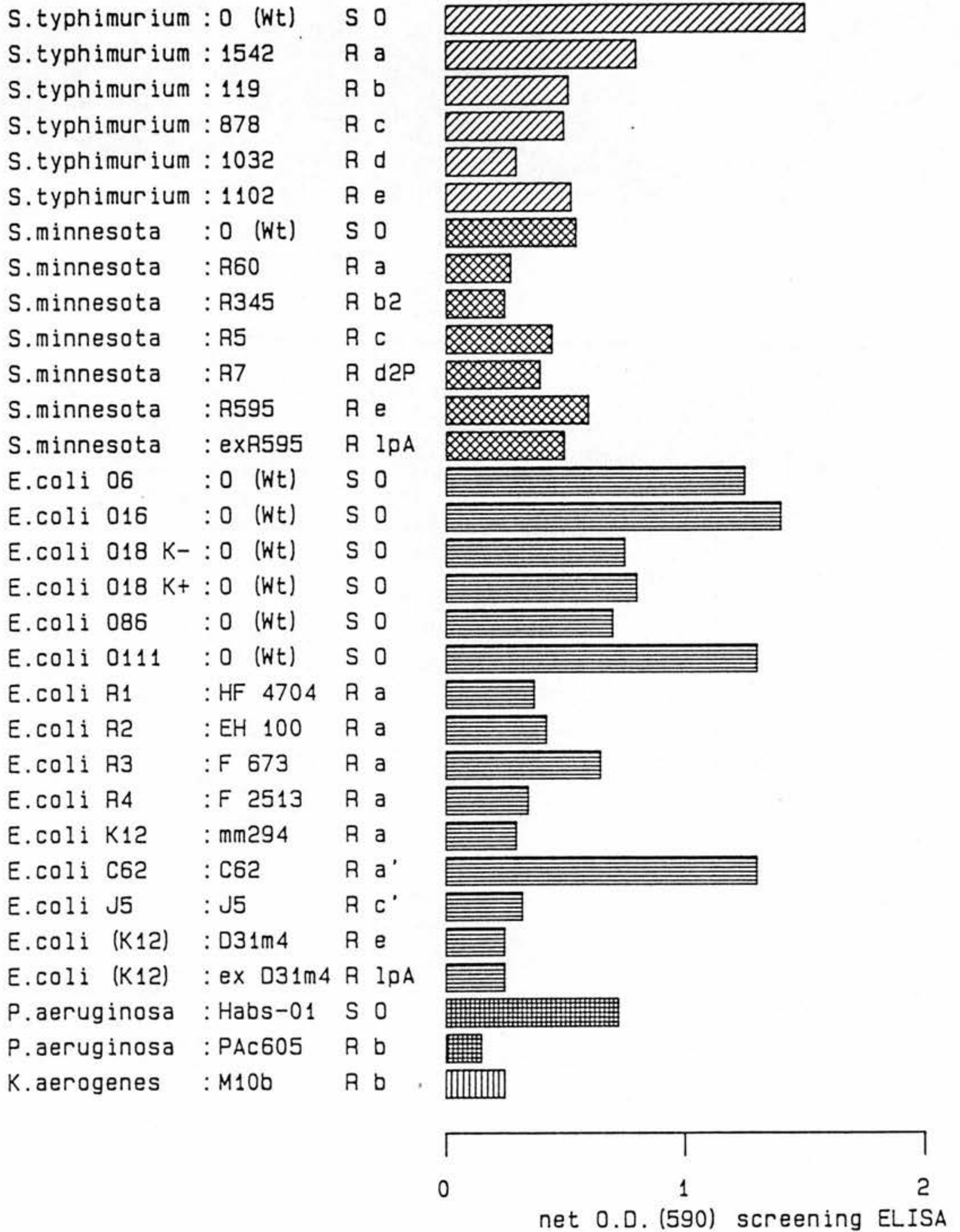
0 1 2

net O.D. (590) screening ELISA

IgG-20

dilution - 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)



IgG-21

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Antigen	Strain	Type
S.typhimurium	: O (Wt)	S O
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: O (Wt)	S O
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli O6	: O (Wt)	S O
E.coli O16	: O (Wt)	S O
E.coli O18 K-	: O (Wt)	S O
E.coli O18 K+	: O (Wt)	S O
E.coli O86	: O (Wt)	S O
E.coli O111	: O (Wt)	S O
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S O
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-22

dilution - 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Organism	Strain	Type
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

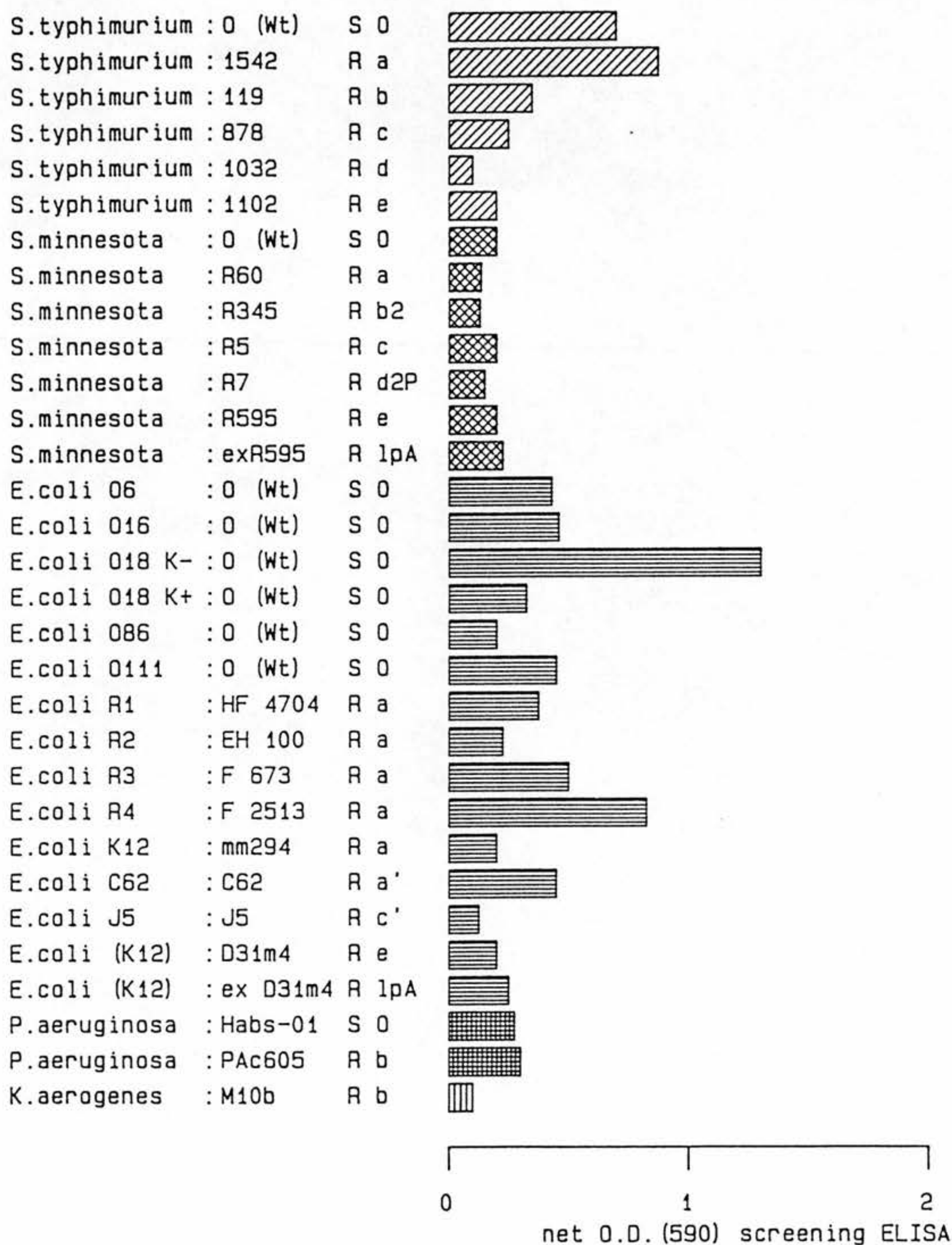
0 1 2

net O.D. (590) screening ELISA

IgG-23

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)



IgG-24

dilution - 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-25

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

0 1 2

net O.D. (590) screening ELISA

IgG-26

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

S.typhimurium	: 0 (Wt)	S	0
S.typhimurium	: 1542	R	a
S.typhimurium	: 119	R	b
S.typhimurium	: 878	R	c
S.typhimurium	: 1032	R	d
S.typhimurium	: 1102	R	e
S.minnesota	: 0 (Wt)	S	0
S.minnesota	: R60	R	a
S.minnesota	: R345	R	b2
S.minnesota	: R5	R	c
S.minnesota	: R7	R	d2P
S.minnesota	: R595	R	e
S.minnesota	: exR595	R	lpA
E.coli 06	: 0 (Wt)	S	0
E.coli 016	: 0 (Wt)	S	0
E.coli 018 K-	: 0 (Wt)	S	0
E.coli 018 K+	: 0 (Wt)	S	0
E.coli 086	: 0 (Wt)	S	0
E.coli 0111	: 0 (Wt)	S	0
E.coli R1	: HF 4704	R	a
E.coli R2	: EH 100	R	a
E.coli R3	: F 673	R	a
E.coli R4	: F 2513	R	a
E.coli K12	: mm294	R	a
E.coli C62	: C62	R	a'
E.coli J5	: J5	R	c'
E.coli (K12)	: D31m4	R	e
E.coli (K12)	: ex D31m4	R	lpA
P.aeruginosa	: Habs-01	S	0
P.aeruginosa	: PAc605	R	b
K.aerogenes	: M10b	R	b

0 1 2

net O.D. (590) screening ELISA

IgG-27

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)

Organism	Strain	
S.typhimurium	: 0 (Wt)	S 0
S.typhimurium	: 1542	R a
S.typhimurium	: 119	R b
S.typhimurium	: 878	R c
S.typhimurium	: 1032	R d
S.typhimurium	: 1102	R e
S.minnesota	: 0 (Wt)	S 0
S.minnesota	: R60	R a
S.minnesota	: R345	R b2
S.minnesota	: R5	R c
S.minnesota	: R7	R d2P
S.minnesota	: R595	R e
S.minnesota	: exR595	R lpA
E.coli 06	: 0 (Wt)	S 0
E.coli 016	: 0 (Wt)	S 0
E.coli 018 K-	: 0 (Wt)	S 0
E.coli 018 K+	: 0 (Wt)	S 0
E.coli 086	: 0 (Wt)	S 0
E.coli 0111	: 0 (Wt)	S 0
E.coli R1	: HF 4704	R a
E.coli R2	: EH 100	R a
E.coli R3	: F 673	R a
E.coli R4	: F 2513	R a
E.coli K12	: mm294	R a
E.coli C62	: C62	R a'
E.coli J5	: J5	R c'
E.coli (K12)	: D31m4	R e
E.coli (K12)	: ex D31m4	R lpA
P.aeruginosa	: Habs-01	S 0
P.aeruginosa	: PAc605	R b
K.aerogenes	: M10b	R b

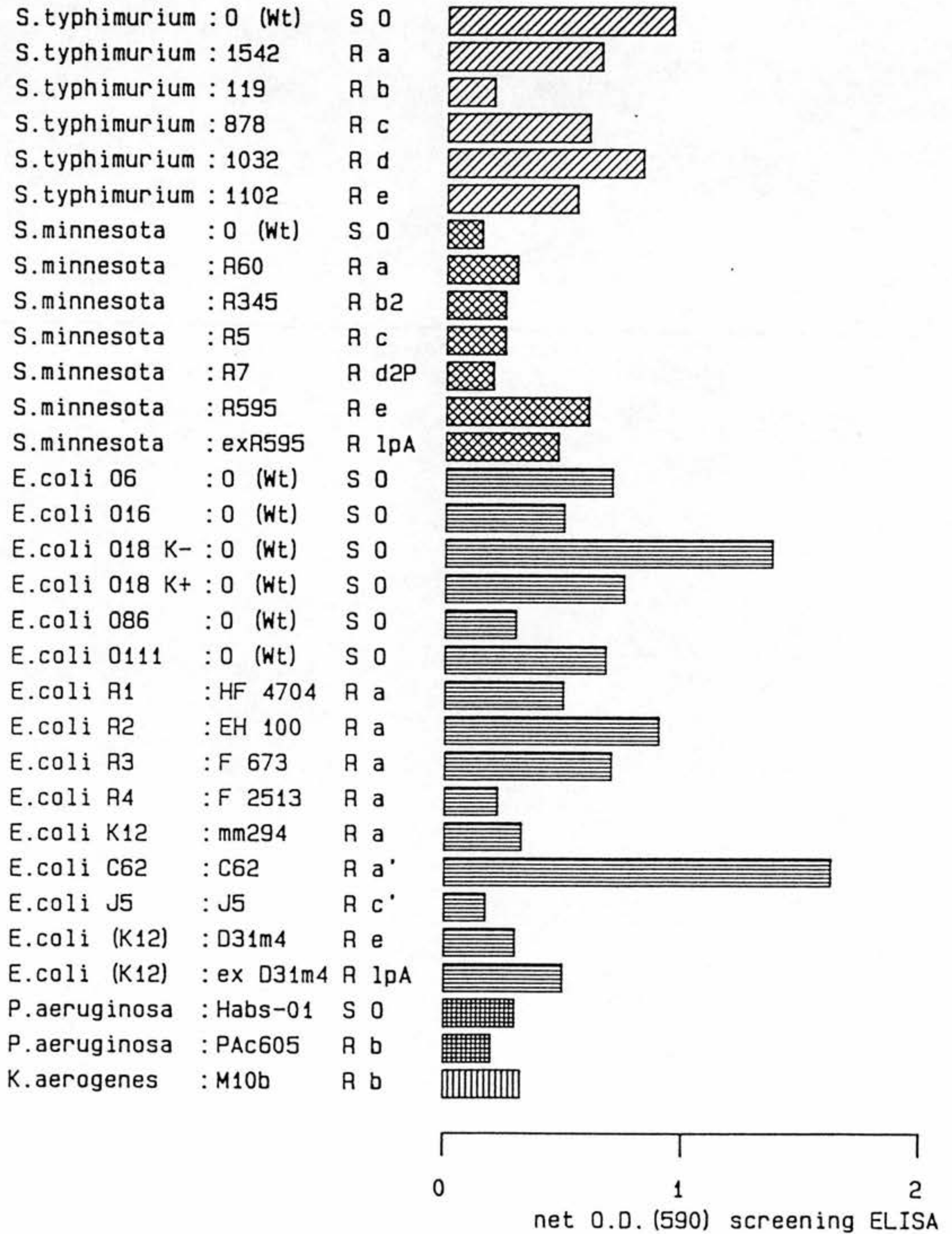
0 1 2

net O.D. (590) screening ELISA

IgG-28

dilution = 1/ 100

ELISA ANTIGENS (LPS-polymyxin complexes)



IgG-29

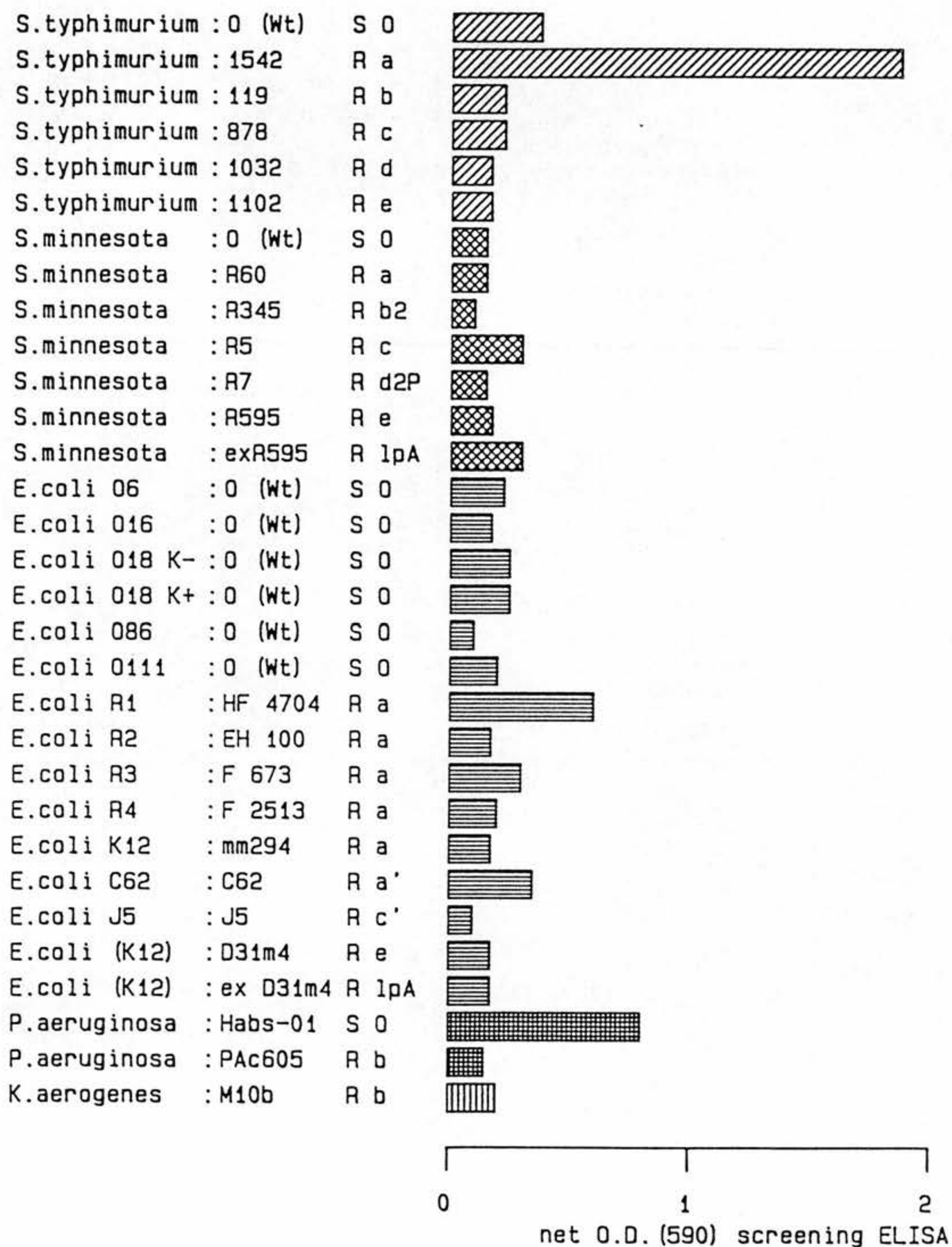
dilution = 1/ 100

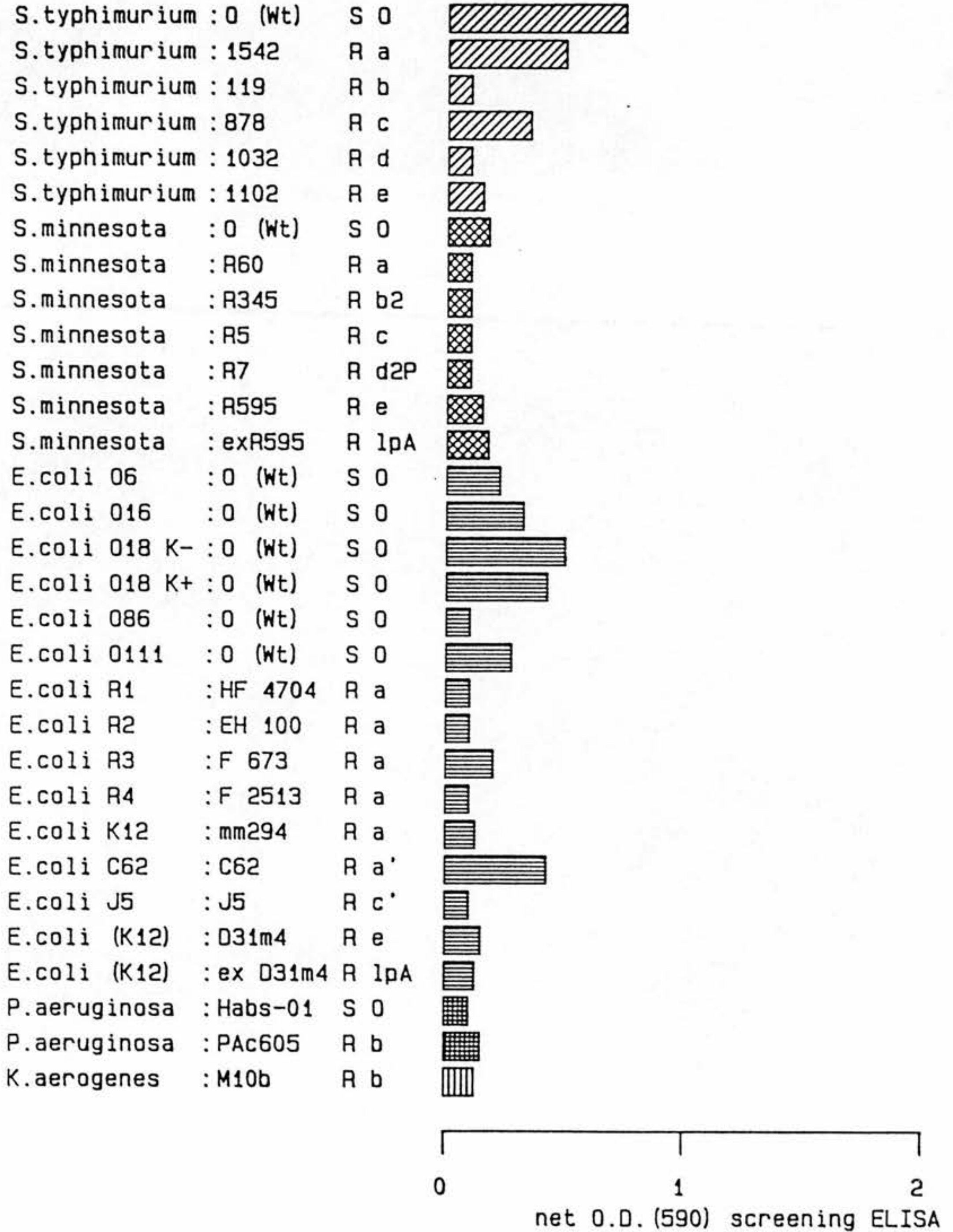
ELISA ANTIGENS (LPS-polymyxin complexes)

Antigen	Strain	Type	Chemotype
S.typhimurium	0 (Wt)	S	0
S.typhimurium	1542	R	a
S.typhimurium	119	R	b
S.typhimurium	878	R	c
S.typhimurium	1032	R	d
S.typhimurium	1102	R	e
S.minnesota	0 (Wt)	S	0
S.minnesota	R60	R	a
S.minnesota	R345	R	b2
S.minnesota	R5	R	c
S.minnesota	R7	R	d2P
S.minnesota	R595	R	e
S.minnesota	exR595	R	lpA
E.coli 06	0 (Wt)	S	0
E.coli 016	0 (Wt)	S	0
E.coli 018 K-	0 (Wt)	S	0
E.coli 018 K+	0 (Wt)	S	0
E.coli 086	0 (Wt)	S	0
E.coli 0111	0 (Wt)	S	0
E.coli R1	HF 4704	R	a
E.coli R2	EH 100	R	a
E.coli R3	F 673	R	a
E.coli R4	F 2513	R	a
E.coli K12	mm294	R	a
E.coli C62	C62	R	a'
E.coli J5	J5	R	c'
E.coli (K12)	D31m4	R	e
E.coli (K12)	ex D31m4	R	lpA
P.aeruginosa	Habs-01	S	0
P.aeruginosa	PAc605	R	b
K.aerogenes	M10b	R	b

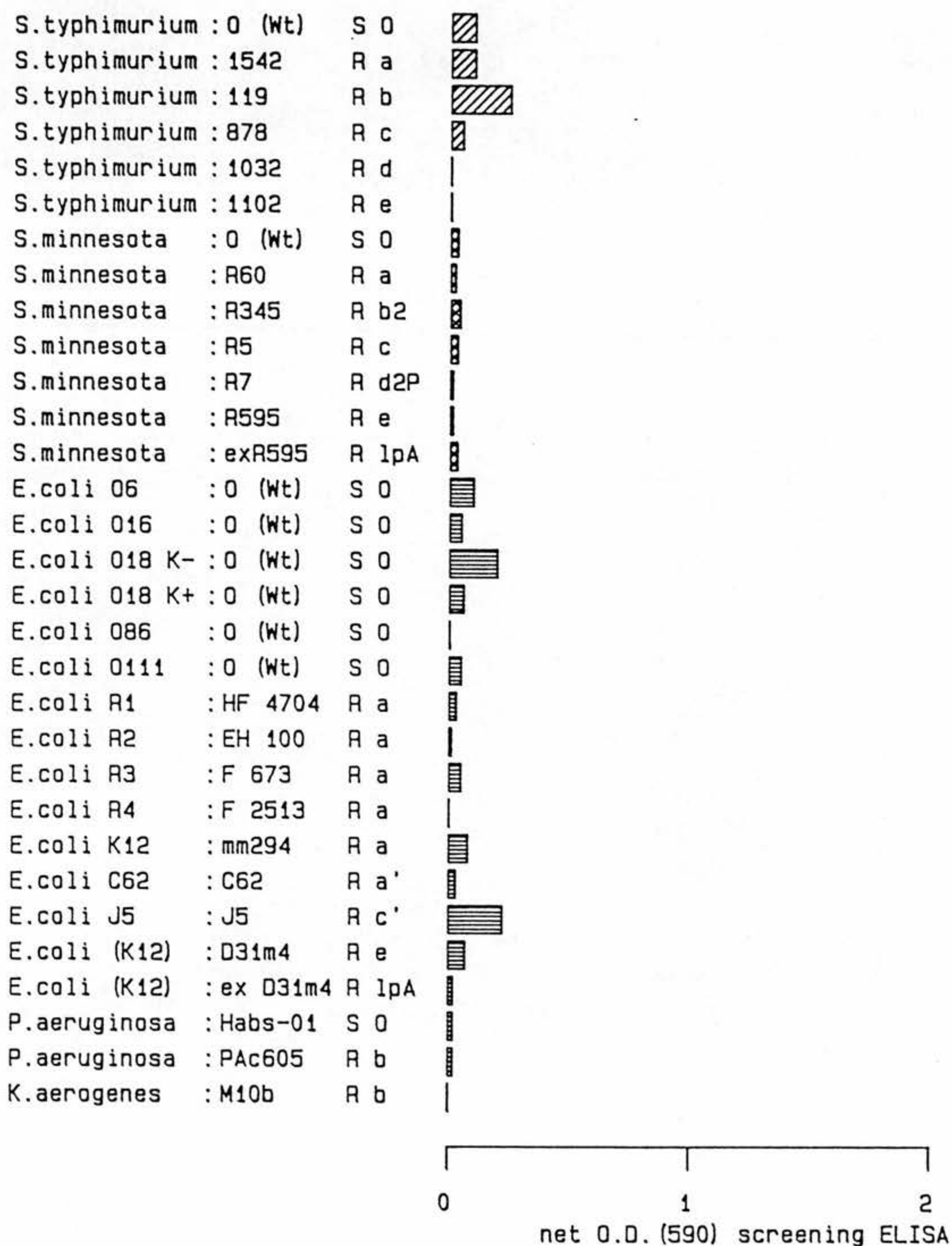
0 1 2

net O.D. (590) screening ELISA

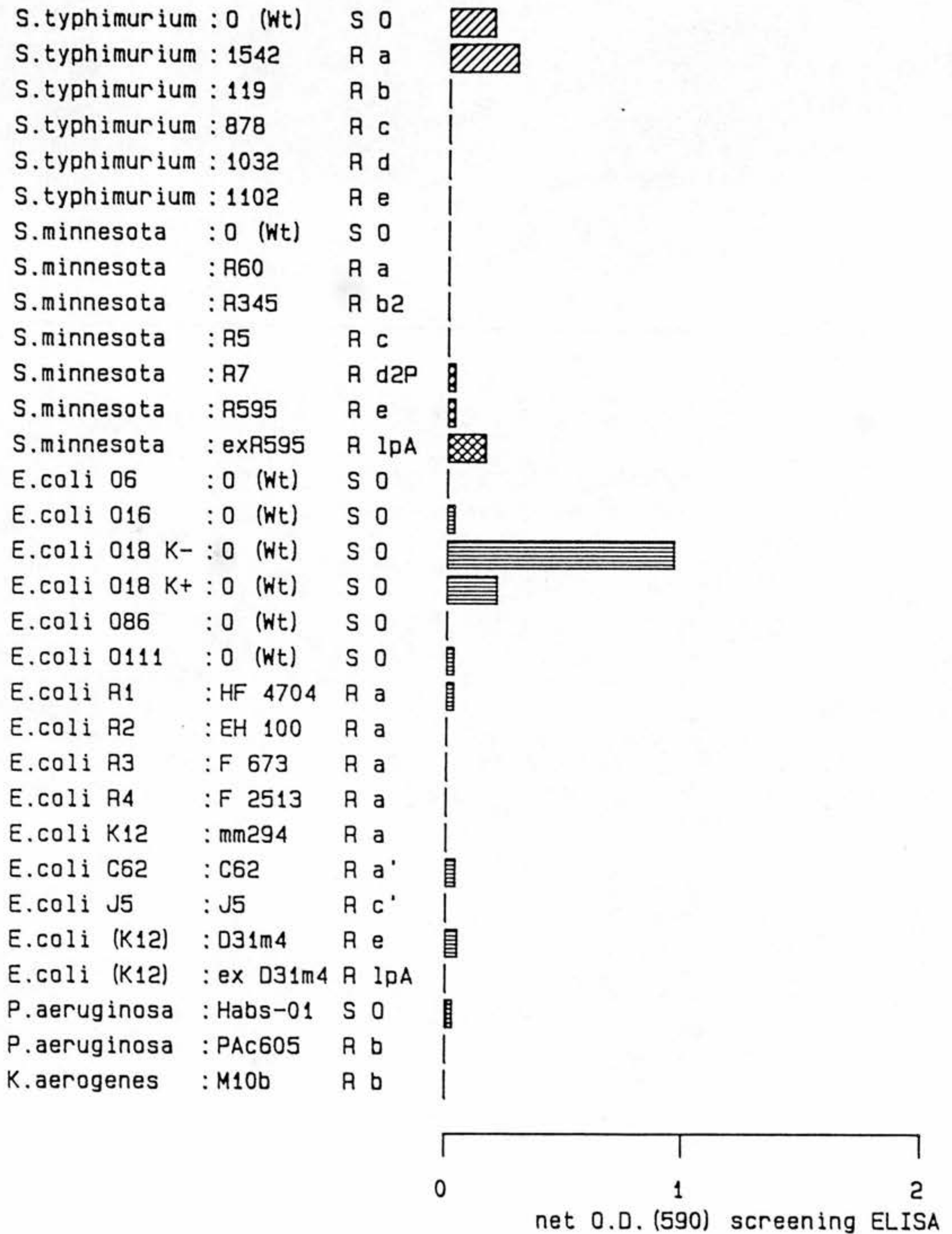

IgG-30
dilution - 1/ 100
ELISA ANTIGENS (LPS-polymyxin complexes)
S.typhimurium : 0 (Wt) S 0
S.typhimurium : 1542 R a
S.typhimurium : 119 R b
S.typhimurium : 878 R c
S.typhimurium : 1032 R d
S.typhimurium : 1102 R e
S.minnesota : 0 (Wt) S 0
S.minnesota : R60 R a
S.minnesota : R345 R b2
S.minnesota : R5 R c
S.minnesota : R7 R d2P
S.minnesota : R595 R e
S.minnesota : exR595 R lpA
E.coli 06 : 0 (Wt) S 0
E.coli 016 : 0 (Wt) S 0
E.coli 018 K- : 0 (Wt) S 0
E.coli 018 K+ : 0 (Wt) S 0
E.coli 086 : 0 (Wt) S 0
E.coli 0111 : 0 (Wt) S 0
E.coli R1 : HF 4704 R a
E.coli R2 : EH 100 R a
E.coli R3 : F 673 R a
E.coli R4 : F 2513 R a
E.coli K12 : mm294 R a
E.coli C62 : C62 R a'
E.coli J5 : J5 R c'
E.coli (K12) : D31m4 R e
E.coli (K12) : ex D31m4 R lpA
P.aeruginosa : Habs-01 S 0
P.aeruginosa : PAc605 R b
K.aerogenes : M10b R b
0
1
2
net O.D. (590) screening ELISA



IgG-31
dilution - 1/ 100
ELISA ANTIGENS (LPS-polymyxin complexes)
S.typhimurium : 0 (Wt) S 0
S.typhimurium : 1542 R a
S.typhimurium : 119 R b
S.typhimurium : 878 R c
S.typhimurium : 1032 R d
S.typhimurium : 1102 R e
S.minnesota : 0 (Wt) S 0
S.minnesota : R60 R a
S.minnesota : R345 R b2
S.minnesota : R5 R c
S.minnesota : R7 R d2P
S.minnesota : R595 R e
S.minnesota : exR595 R lpA
E.coli 06 : 0 (Wt) S 0
E.coli 016 : 0 (Wt) S 0
E.coli 018 K- : 0 (Wt) S 0
E.coli 018 K+ : 0 (Wt) S 0
E.coli 086 : 0 (Wt) S 0
E.coli 0111 : 0 (Wt) S 0
E.coli R1 : HF 4704 R a
E.coli R2 : EH 100 R a
E.coli R3 : F 673 R a
E.coli R4 : F 2513 R a
E.coli K12 : mm294 R a
E.coli C62 : C62 R a'
E.coli J5 : J5 R c'
E.coli (K12) : D31m4 R e
E.coli (K12) : ex D31m4 R lpA
P.aeruginosa : Habs-01 S 0
P.aeruginosa : PAc605 R b
K.aerogenes : M10b R b
0
1
2
net O.D. (590) screening ELISA



IgG-32
dilution - 1/ 100
ELISA ANTIGENS (LPS-polymyxin complexes)
S.typhimurium : 0 (Wt) S 0
S.typhimurium : 1542 R a
S.typhimurium : 119 R b
S.typhimurium : 878 R c
S.typhimurium : 1032 R d
S.typhimurium : 1102 R e
S.minnesota : 0 (Wt) S 0
S.minnesota : R60 R a
S.minnesota : R345 R b2
S.minnesota : R5 R c
S.minnesota : R7 R d2P
S.minnesota : R595 R e
S.minnesota : exR595 R lpA
E.coli 06 : 0 (Wt) S 0
E.coli 016 : 0 (Wt) S 0
E.coli 018 K- : 0 (Wt) S 0
E.coli 018 K+ : 0 (Wt) S 0
E.coli 086 : 0 (Wt) S 0
E.coli 0111 : 0 (Wt) S 0
E.coli R1 : HF 4704 R a
E.coli R2 : EH 100 R a
E.coli R3 : F 673 R a
E.coli R4 : F 2513 R a
E.coli K12 : mm294 R a
E.coli C62 : C62 R a'
E.coli J5 : J5 R c'
E.coli (K12) : D31m4 R e
E.coli (K12) : ex D31m4 R lpA
P.aeruginosa : Habs-01 S 0
P.aeruginosa : PAc605 R b
K.aerogenes : M10b R b
0
1
2
net O.D. (590) screening ELISA



IgG-33
dilution - 1/ 100
ELISA ANTIGENS (LPS-polymyxin complexes)
S.typhimurium : 0 (Wt) S 0
S.typhimurium : 1542 R a
S.typhimurium : 119 R b
S.typhimurium : 878 R c
S.typhimurium : 1032 R d
S.typhimurium : 1102 R e
S.minnesota : 0 (Wt) S 0
S.minnesota : R60 R a
S.minnesota : R345 R b2
S.minnesota : R5 R c
S.minnesota : R7 R d2P
S.minnesota : R595 R e
S.minnesota : exR595 R lpA
E.coli 06 : 0 (Wt) S 0
E.coli 016 : 0 (Wt) S 0
E.coli 018 K- : 0 (Wt) S 0
E.coli 018 K+ : 0 (Wt) S 0
E.coli 086 : 0 (Wt) S 0
E.coli 0111 : 0 (Wt) S 0
E.coli R1 : HF 4704 R a
E.coli R2 : EH 100 R a
E.coli R3 : F 673 R a
E.coli R4 : F 2513 R a
E.coli K12 : mm294 R a
E.coli C62 : C62 R a'
E.coli J5 : J5 R c'
E.coli (K12) : D31m4 R e
E.coli (K12) : ex D31m4 R lpA
P.aeruginosa : Habs-01 S 0
P.aeruginosa : PAc605 R b
K.aerogenes : M10b R b
0
1
2
net O.D. (590) screening ELISA

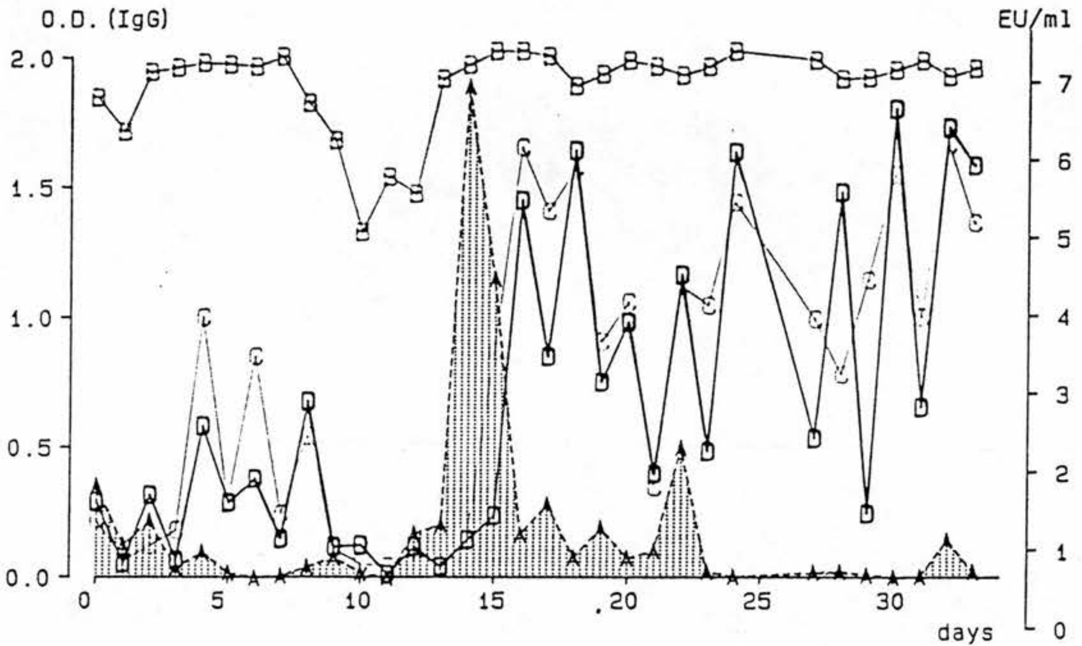
APPENDIX 2

Key to Appendix 2.

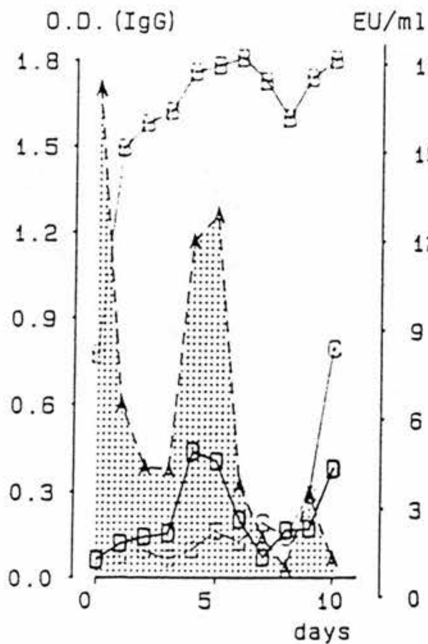
EU/ml:Endotoxin units/ml

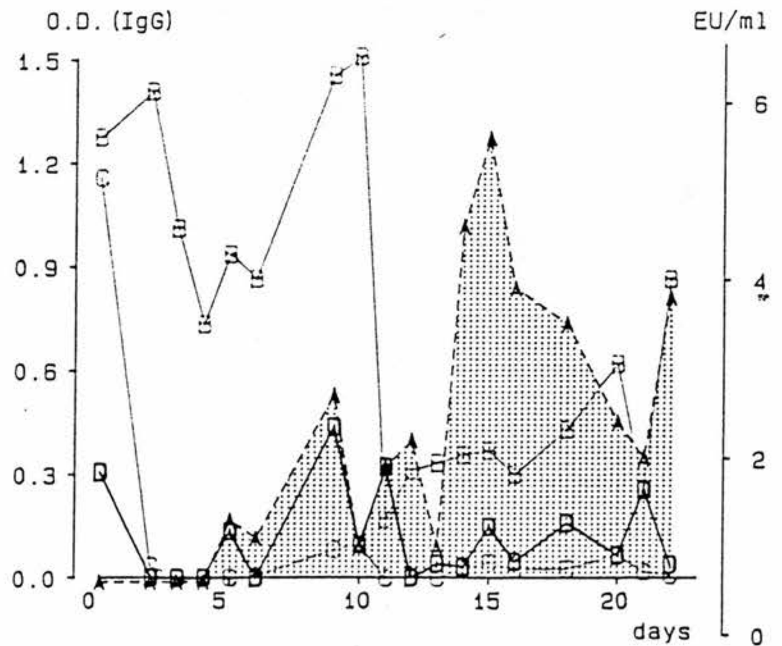
Sm-Re: *S. minnesota* R595 LPS
S.min lipid A: *S. minnesota* lipid A
St-Ra: *S. typhimurium* R1542 LPS
St-Rc: *S. typhimurium* R878 LPS
St-Re: *S.typhimurium* R1102 LPS
Ec lipid A:*E. coli* K12 lipid A

Serum Endotoxin & IgG anti-LPS-cores

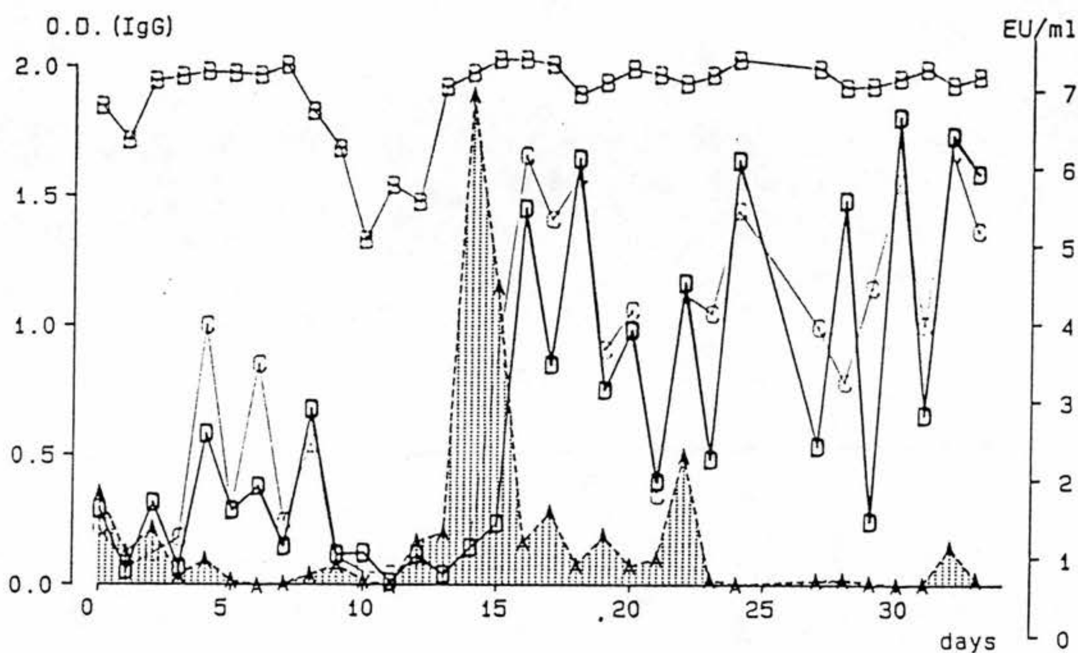


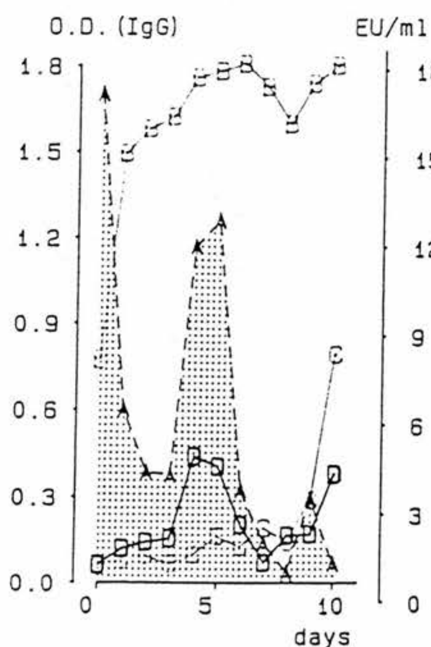
KEY: A=EU/ml: B=St-Ra: C=St-Rc: D=St-Re:

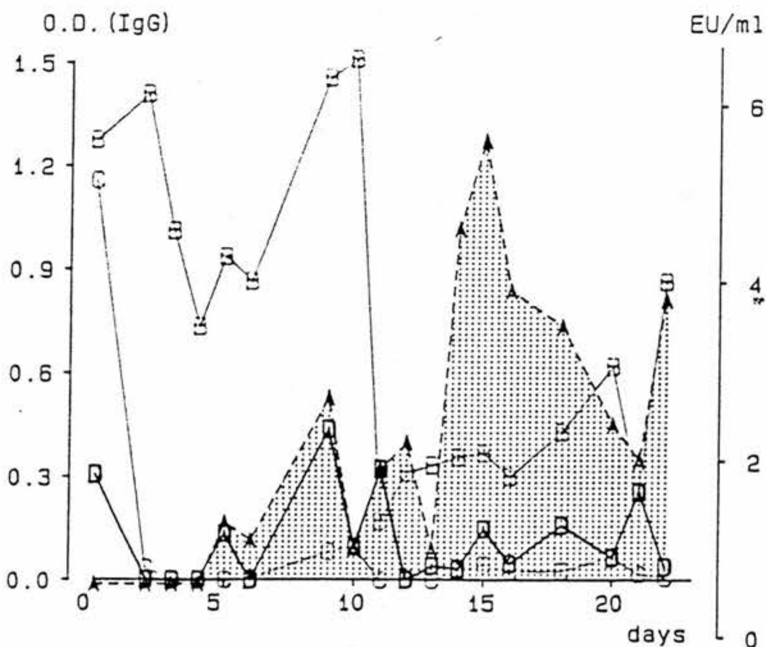




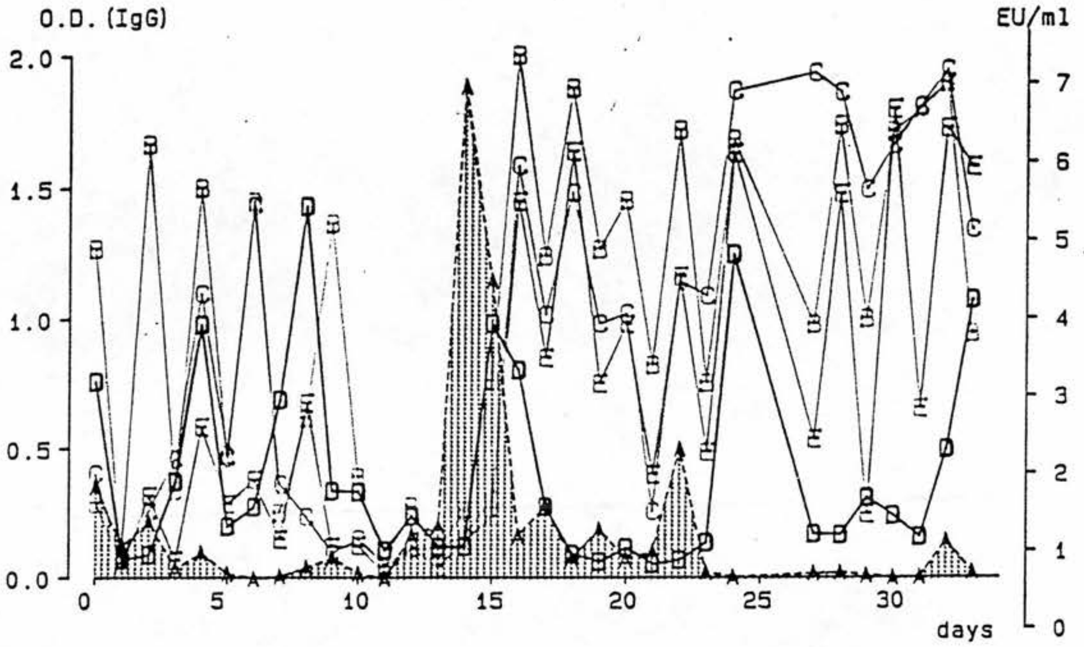
Serum Endotoxin & IgG anti-LPS-cores







Serum Endotoxin & IgG anti-LPS



KEY: A=EU/ml: B=S.min lipid A: C=Ec lipid A: D=Sm-Re: E=St-Re: